1994

Study on the Neural Cell Adhesion Molecules in Heart Development of Chicken

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by

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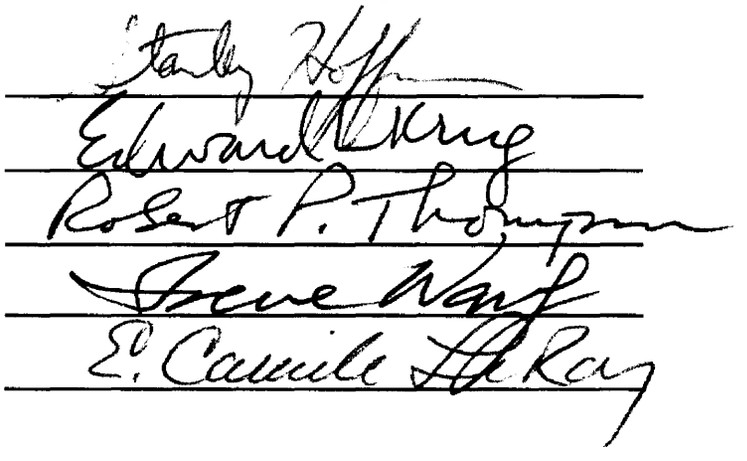
A Thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the College of Graduate Studies.

Molecular and Cellular Biology and Pathobiology Program

1994

Approved by :

Chairman, Advisory Committee



ACKNOWLEDGMENT

To make a seed root to soil, develop into branches and leaves, and it become to bloom and produce fruits, a myriad of surroundings have existed.

Without my family's unconditional love and support, I would not be the person I am today. My parents have provided all the atmosphere for me to grow as an independent creature. They taught me the wisdom to balance life and to free the spirit, in addition to oriental virtue. I thank to my beautiful sister, Minae, a graduate student, who has been my mental companion along the way. I also like to express my gratitude to all of my close friends here, who truly have been there at any time for me.

I was very fortunate to have Dr. Stanley Hoffman as my supervisor, who sincerely is a dedicated scientist. His ingenuity, creativity, and flexibility have deeply influenced me. He has patiently watched and helped me out so that I could be at least a useful individual. I will miss the moments in the lab when our dreams and imaginations of science went beyond the scope of daily routine.

I extend my deep appreciation to my thesis committee, Dr. Carwile LeRoy, Dr. Edward Krug, Dr. Robert Thompson, and Dr. Irene Wang, who have helped and encouraged each of my academic steps. I specially thank you to Dr. Yukiko Sugi and Dr. Roger Markwald, collaborators, for their excellent guidance through the world of heart development. I want to thank lab

colleagues, Sandra Dutton, Teresa Pickens, Kathy Zanin, David Everman, and Michael Bonner, as well as to Dr. Heidi Ernst and Dr. Andrey Torkin. They were the ones I could chat, complain, and ask anything for 24 hours a day. I also like to acknowledge Sue Burrows for her wonderful photographic work, Thomas Trusk for his invaluable assistance in laser confocal microscopy and image processing. I would like to thank to Dr. Barry Ledford, the student adviser of MCBP program, for his heartfelt guide and advices.

My sincere appreciation would extend to Dr. S. D. Park, my former mentor in Seoul National Univ, as well. He taught how to swim in this science world at my very start.

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LIST OF ABBREVIATIONS

ABTS	:	2, 2'-Azinobis (3-ethylbenzoothiazoline-6-sulfonic acid) diammonium salt
BCIP	:	bromochloroindoyl phophate
BF	:	buffer
Br	:	brain
BSA	:	bovine serum albumin
Ca^{++}	:	calcium ion
CAM	:	cell adhesion molecule
CNBr	:	cyanogen bromide
DEAE	:	diethylaminoethyl
DTT	:	dithiothreitol
ECT	:	endocardial cushion tissue
EDTA	:	ethylene-diamine tetraacetic acid
EGTA	:	(ethylene-dioxy) diethylenedinitro tetraacetic acid
ELISA	:	enzyme-linked immunoabsorbent assay
F-actin	:	filamentous actin
FITC	:	fluorescein isothiocyanate
GPI	:	glycosyl-phosphatidylinositol
H_2O_2	:	hydrogen peroxide
HNK-1	:	human natural killer-1
HPLC	:	high pressure liquid chromatography
HRP	:	horseradish peroxidase
Ht	:	heart
IgG	:	immunoglobulin G
kb	:	kilobase
kD	:	kilodalton

KLH : keyhole limpet hemocyanin

ld : large cytoplasmic domain

mb : membrane

mM : milimolar

mRNA : messenger RNA

µg : microgram

µm : micrometer

MSD : muscle specific domain

NaCl : sodium chloride

NBT : nitroblue tetrazolium

NCAM : neural cell adhesion molecule

NP40 : nonidet P-40

OD : optical density

OTG : octylthioglucopyranoside

PBS : phosphate buffered saline

PCR : polymerase chain reaction

PIPLC : phosphatidylinositol-specific phospholipase C

PMSF : phenylmethylsulfonyl fluoride

PSA : polysialic acid

rpm : revolution per minute

RT-PCR : reverse transcriptase-based polymerase chain reaction

sd : small cytoplasmic domain

SDS : sodium dodecyl sulfate

SDS-PAGE : SDS containing polyacrylamide gel electrophoresis

Ser : serine

ssd : small surface domain

SPDP : N-succinimidyl 3-(2-pyridyl dithio) propionate

TBS : tris buffered saline

Thr : threonine

TRITC : tetramethylrhodamine isomer R

VASE : variable alternatively spliced exon

ABSTRACT

Cell adhesion molecules mediate specific cell-cell interactions during embryogenesis and tissue formation. Among cell-cell adhesion molecules (CAMs), we have been particularly interested in the neural cell adhesion molecule (NCAM) which is the prototypical member of the family of Ca^{++} - independent CAMs. Previous studies of NCAM cDNAs have revealed an alternatively spliced set of small exons (12A, 12B, 12C, 12D) that encode a region in the extracellular portion of the molecule known as the muscle specific domain (MSD). The entire MSD region can be expressed in skeletal muscle, heart, and skin; only exons 12A and 12D have been found in brain. These studies, however, did not reveal which NCAM polypeptides contain the MSD region or the immunohistochemical distribution of these NCAM molecules. To address these questions, we prepared antibodies against the oligopeptides encoded by exons 12A and 12B and by exons 12C and 12D and used these antibodies to study the forms of NCAM containing the MSD region expressed during embryonic chicken heart development. These antibodies recognize certain forms of NCAM found in the heart but do not recognize brain NCAM. In the heart, each of the splice variants of NCAM (ld, sd, and ssd) that differ in their mode of attachment to the plasma membrane or in the size of their cytoplasmic domain is expressed in a form containing and in a form lacking the MSD region. No microheterogeneity is observed in

the size of NCAM molecules containing the MSD region, even at the level of CNBr fragments, suggesting that exons 12A-D are expressed as a single unit. We have identified six different isoforms of heart NCAM which exhibit changing temporal and spatial patterns of expression during development: the three forms of NCAM, ld, sd, and ssd, can also be expressed in a form containing the MSD sequence. Depending on the site and the stage of development, the percentage of NCAM molecules containing the MSD region can vary from nearly 0 to 100 percent. In general, this percentage increases during development. In immunohistochemical studies of hearts from stage 18 embryos, forms of NCAM containing the MSD region colocalized with Z-discs. No other adhesion molecules were found in this distribution at this early stage of development. Studies on isolated cells in vitro demonstrate that the colocalization with Z discs of NCAM molecules containing the MSD region does not depend on cell-cell contact and raise the possibility that this form of NCAM is involved in cell-extracellular matrix interactions. The association of NCAM molecules containing the MSD region with Z discs suggests that this form of NCAM is involved in early myofibrillogenesis.

INTRODUCTION

NCAM, The Neural Cell Adhesion Molecule

The cellular and molecular processes of development within an organism involve series of biological changes. Initiation or progression of each developmental event such as cell migration, proliferation, or tissue differentiation is precisely programmed to be coordinated in time and space. It has been demonstrated that cell adhesions are important contributors to the estabishment of form and pattern during development (17, 74). In general, adhesion occur by two ways, cell-cell interactions or cell-extracellular matrix interactions. These interactions are based on specific adhesion molecules, cell adhesion molecules (CAMs) and cell-substrate adhesion molecules (SAMs). CAMs are membrane receptors that mediate cell-to-cell adhesion by their interactions at the cell surface. CAMs are largely divided into two groups according to their Ca^{++}-dependence : the Ca^{++}-dependent adhesion molecules known as cadherins and the Ca^{++}-independent adhesion molecules including the neural cell adhesion molecule (NCAM) (17, 32, 74). SAMs mediate cell-to-extracellular matrix (ECM) adhesion by the interactions of extracellular matrix proteins with their cell surface receptors (14, 18, 20, 24). At the functional level, adhesion molecules specifically control cell behavior both directly and

indirectly. That is, adhesion molecules directly mediate the cell migration and maintain tissue borders at the surface of cells, and at the same time adhesion molecules indirectly affect the fate of cells through the transmembrane signal transduction mechanisms their binding initiates (11, 17, 33, 36, 65, 70).

Among the cell adhesion molecules, NCAM has been of particular interest to us. NCAM mediates cell-cell adhesion both by a homophilic mechanism (31, 52, 61, 62), i.e., NCAM to NCAM, and by heterophilic mechanisms involving the interaction of a heparin-binding domain in NCAM with heparan sulfate proteoglycans (57) or the interaction of NCAM with neurocan, a chondroitin sulfate proteoglycan (54). NCAM is expressed in a number of cell types including neuron, skeletal muscle, heart, kidney, liver, gizzard, ovary, skin, testis, and lymphocyte (2, 10, 65). It is present abundantly in various tissues from very early embryos through adults (2, 17). It plays major morphoregulatory roles in guiding tissue formation and differentiation (2, 17). NCAM is structural diverse (46). It contains a large number of unusual polysialic acid (PSA) side chains that are important for its adhesive function (1, 9, 17, 32).

Structural Diversity of the NCAM Polypeptide

NCAM was the first cell adhesion molecule to be identified. It is a cell surface glycoprotein containing large amonts of polysialic acid (PSA). PSA is

composed of unique α-2,8-linked oligosaccharides that are rare in other molecules (9, 21, 60). One type of NCAM heterogeneity is due to differential polysialylation. Polysialylation modulates NCAM adhesion (1, 31) and the level of polysialylation of NCAM correlates with development of an organism (13, 21, 31, 32).

Another type of heterogeneity is found in the NCAM poypeptide. NCAM belongs to the IgG superfamily, containing immunoglobulin (IgG)-homologous domains. The extracellular structure is well conserved in all NCAM species: there are five IgG-domains and two fibronectin type III homologous repeats (9, 32) (Figure 1). In contrast, the cytoplasmic portion of NCAM exhibits a great deal of structural heterogeneity. One form of NCAM, ssd (small surface domain), is covalently linked to the plasma membrane via glycolipid glycosyl-phosphatidylinositol (GPI); two integral membrane forms, ld (large domain) and sd (small domain), differ in the size of their cytoplasmic domains (3, 27, 28, 46, 62). These various forms of NCAM are produced by alternative mRNA splicing from a single gene. Studies have shown that forms of NCAM vary according to the tissue type. The ld form is a major NCAM species in brain tissue, while sd or ssd are predominant in skeletal muscle. It has been predicted that tissue-specific modes of alternative splicing exist for NCAM synthesis. However, the molecular mechanism through which splicing occurs is still not known.

Schematic Model of NCAM Splice Variants

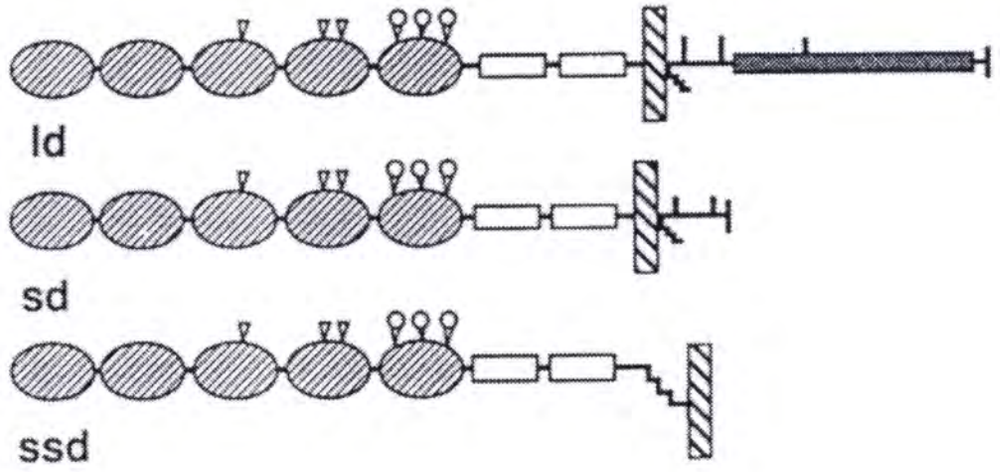

Figure 1. Models of three major NCAM Splice Variants.

The structural diversity of NCAM largely divides into three groups as illustrated. All three forms are similar in their extracellular structure: five IgG homologous domains (hatched oval) at the N-terminus are followed by two fibronectin-like repeats (□). Unique polysialic acid side chains (♀) are covalently bound to the IgG homologous domains. The NCAM splice variants differ in their mode of cell membrane attachment or in the truncation of transmembrane and/or cytoplasmic region. While the ld (large domain) form contains an entire sequence encoded by all 19 exons, the sd (small domain) lacks the exon 18 (gray box)-encoded region, or the ssd (small surface domain) is only GPI-linked (staggered line) to the cell membrane (hatched box) with transmembrane and cytoplasmic regions exclusively truncated (see Figure 3 for more details).

Figure source: Edelman & Crossin. 1991. "Cell adhesion molecules: implications for a molecular histology". Annu. Rev. Biochem.

In addition, two small inserts in the NCAM polypeptide have been identified. They are spliced into two distinct locations in the extracellular portion of NCAM by alternative mRNA splicing. One is the VASE (variable alternatively spliced exon) insert that is present between the sequences encoded by exons 7 and 8 and is found in both brain and heart (64, 66, 67, 79). The other is the so-called muscle-specific domain or MSD region encoded by four small exons (12A, 12B, 12C, 12D) that are found between exons 12 and 13 (51, 72) (Figure 2, 3). This insert is expected to be localized between two fibronectin homologous domains in the extracellular portion of NCAM. Because no reagents that recognize MSD polypeptide have been prepared previously, little was known of the distribution, structure, and function of MSD polypeptides.

Molecular biology study based on RT-PCR (reverse transcriptase-based polymerase chain reaction) has suggested that the MSD is expressed in a variety of splice combinations of these four small exons (56). For example, only exons 12A and 12D from this region are expressed in the brain (26, 55, 64), all four of these small exons are expressed in heart, skeletal muscle, and skin (15, 43, 51). While the sequence of these four small exons is highly conserved between humans, rats, and mice, two of the four chicken exons (12A and 12B) are very different in sequence from their mammalian counterparts (51).

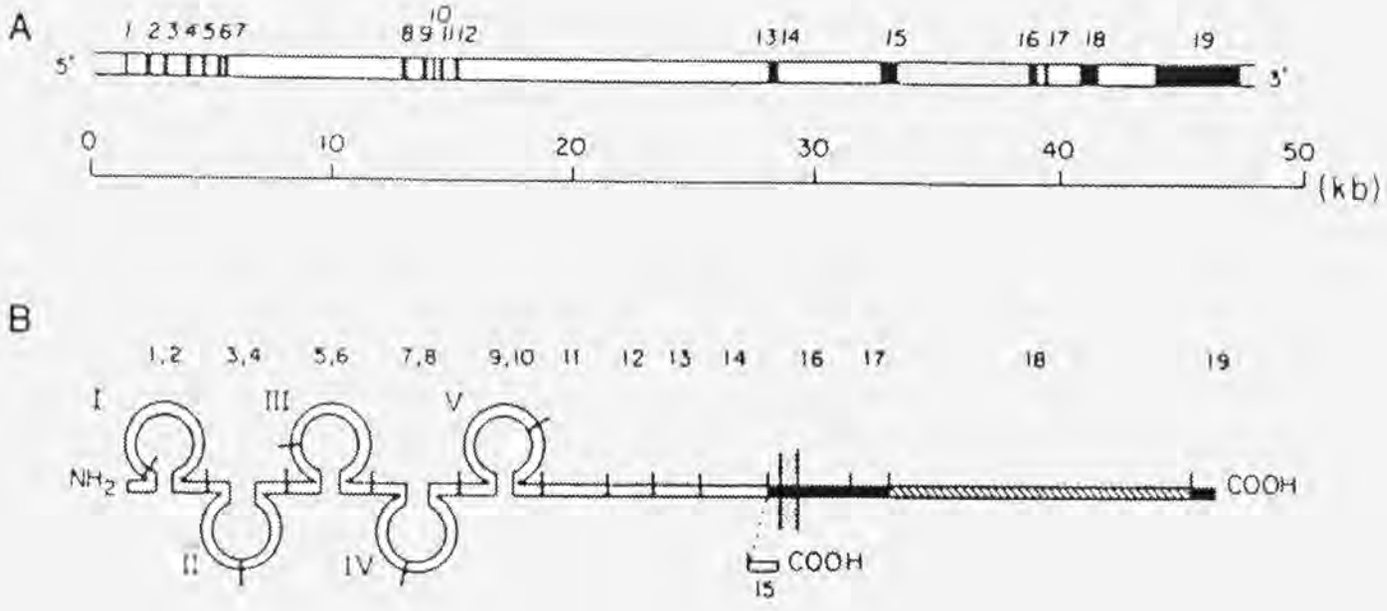

Figure 2. Structure of the NCAM Gene and an Intact NCAM Polypeptide.

(A) The size and location of introns (□) and major exons (■) are indicated in the map. (B) The predicted structure of an intact NCAM polypeptide (B). The structure of ld (large cytoplasmic domain) species is illustrated. ld is the largest splice variant of NCAM which is encoded by the most of major 19 exons. The immunoglobulin homologous regions are shown (loops I-V). Alternative mRNA splicing can generate the other two major variants. The sd (small domain) transmembrane variant lacks the exon 18 sequence. The GPI-linked ssd (small surface domain) variant contains an extra sequence encoded by exon 15. kb = kilobase.

Figure source: Cunningham, B. A. 1988. "The structure and function of cell adhesion molecules". Advances in Cell Biol.

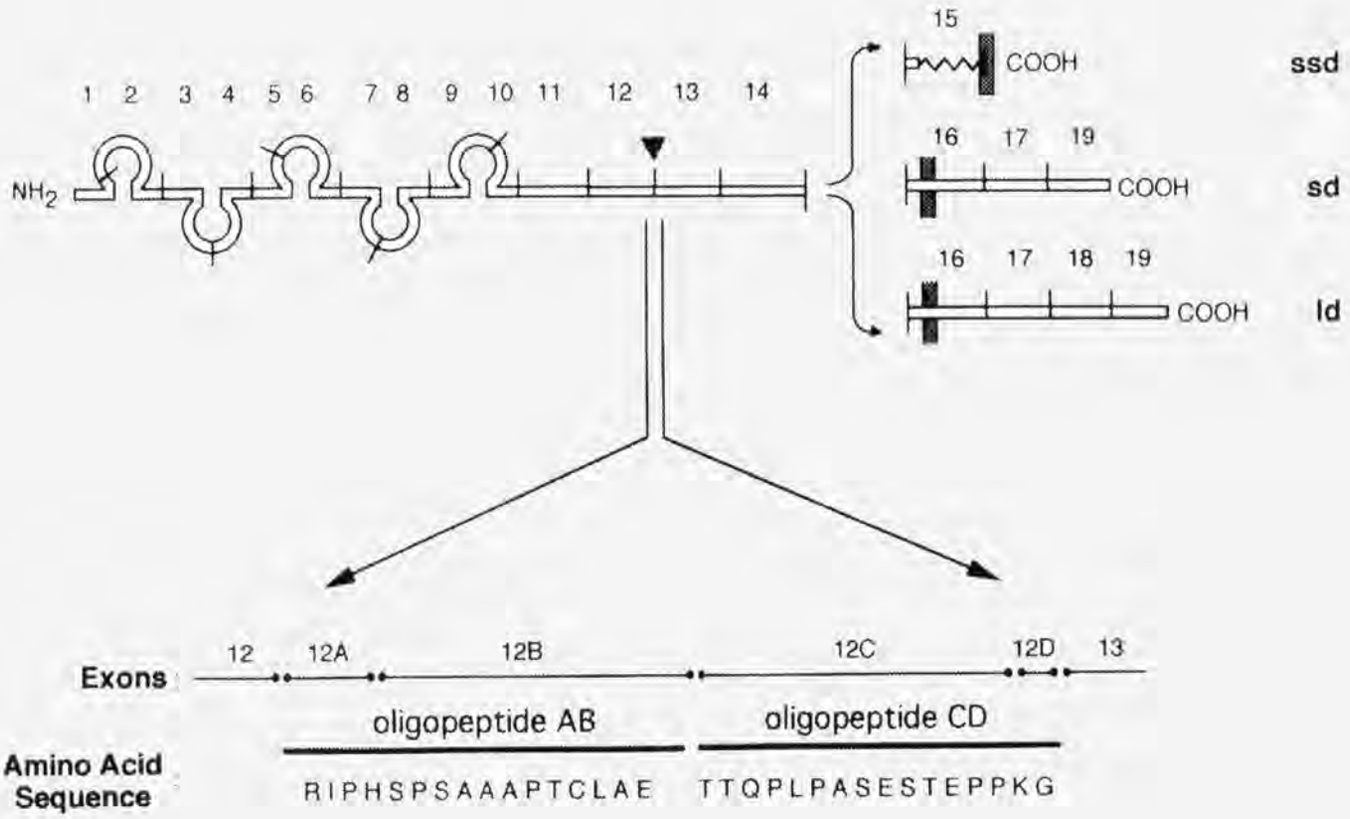

Figure 3. Schematic model of forms of NCAM produced by alternative mRNA splicing in the MSD and membrane attachment regions.

Exon numbers encoding the various regions within the molecule are indicated. ld, sd, and ssd forms differ in their attachment to the membrane through the expression of transmembrane and cytoplasmic exons 16, 17, 18, and 19 or through the expression of exon 15 encoding a peptide that becomes covalently linked to GPI. The MSD region comprised of exons 12A, 12B, 12C, and 12D is expanded, the sequences of the peptides from this region used to immunize rabbits are shown in single-letter code.

In human skeletal muscle, the MSD comprises 37 amino acids and is encoded by at least three small exons of 15, 48, 42 bp at the junction of exons 12 and 13 (15, 72). Similarly, chicken MSD is 31 amino acid sequence. This heart insert is encoded by a set of four exons, 12 A, B, C, and D of 15, 33, 42, and 3 bp, at the same genetic location (15, 51, 56, 64).

Interestingly, mRNA encoding the MSD region of NCAM has been detected only in some tissues. The MSD exists in NCAM molecules produced in skeletal muscle, heart, and skin NCAM, but not in brain, gizzard, or liver. This suggests that the expression of MSD sequence is very tissue-specific (15, 26, 43, 75, 78).

Structural *versus* Functional Diversity of NCAM

NCAM is a multifunctional adhesion molecule which can bind to more than one specific ligand. NCAM operates via both homophilic (31, 53, 61, 62) and heterophilic binding mechanisms. That is, the NCAM binds to NCAM on apposing cells (31) as well as to other ligands such as heparin or heparan sulfate proteoglycan (57). Specific domains within NCAM mediating various types of NCAM interactions have begun to be uncovered by mutational analyses or by domain-targeted inhibition studies (53). However, very little is known about the importance of small structural motifs, VASE and MSD, that are present in NCAM. Functional studies on splice variants of NCAM suggest that the expression of the VASE insert inhibits neurite

outgrowth, while the MSD region has no effect on it (16). On the other hand, recent experiments suggest that the MSD insert found in the 125 kD NCAM played a role in myoblast fusion during myogenesis (49). These observations strongly suggest that inclusion of a small peptide motif significantly affects the NCAM function. Structural features of the MSD include consecutive proline residues as well as O-linked carbohydrate acceptor sites (75).

The mode of association of NCAM with the cell membrane may also have functional consequences. A transfection study has shown that the GPI-linked ssd form of NCAM is targeted to the apical surface of polarized epithelial cells differently than the transmembrane form (50). Others demonstrate that transmembrane forms of NCAM downregulate the secretion of a matrix metalloproteinase, gelatinase, while the GPI-linked form (125 kD) does not have this effect (19).

Our studies have focused on the expression of the MSD insert in NCAM species. We have carefully examined the structural and functional alteration of NCAM in the presence of a MSD motif. We also have compared the spatial or temporal distribution of NCAM species in heart tissue and related their potential function to heart morphogenesis.

NCAM Function and Heart Development

There are many stages in early heart development when changes in the expression or function of cell-cell adhesion molecules may mediate or stablize

changes in morphology or may influence other developmental process. The best-known example of NCAM-mediated differentiation in the heart occurs during the formation of valves.

Initially, the primitive heart (precardiac mesodermal epthelium) appears as paired primordia (6, 59). NCAM and N-cadherin are expressed on almost all of these cells (7, 40). Some of these precardiac cells deadhere as they downregulate expression of these adhesion molecules, convert into mesenchymal cells, and migrate into the basement membrane. The primitive heart primorida then fuse to form one double-layered tube consisting of an inner and outer epithelia (the endocardium and myocardium, respectively) separated by a middle acellular basement membrane known as the cardiac jelly.

The transformation of the heart from a simple tube to a four-chambered structure containing valves involves a second epithelial-mesenchyme transformation. That is, the ECT arises from cells of the endocardial endothelium that de-adhere, convert into mesenchymal cells, and migrate into the cardiac jelly (the epithelial-mesenchymal transformation) (38, 41). NCAM is continuously expressed on all cells of the myocardium and endocardial endothelium; in contrast, N-cadherin is expressed only in the myocardium at this stage of heart development (40). Concomitantly with their de-adhesion, migrating ECT cells down-regulate their expression of NCAM (7, 45) as assayed immunohistochemically (7, 45, 71). Later in development, the growing cushion tissue pads fuse in a manner

that subdivides the flow of blood through the heart into two channels. It is therefore likely that the regulation of NCAM expression or function plays a major role in mediating this sequence of de-adhesion and adhesion that occur during the epithelial-mesenchymal transformation and the fusion of cushion tissue pads.

While cDNA sequence data have demonstrated the existence of NCAM molecules containing the MSD region, the presence of these inserts in particular NCAM polypeptides and their immunohistological distribution in the tissue have not been examined. This was due to the absence of specific antibody probe that can selectively identify the MSD peptide inserts found in NCAM subspecies. This thesis, therefore, emphasizes two specific aims. The first aim is to identify and characterize NCAM species containing the MSD at the level of protein. This requires production of an antibody specifically directed against the MSD sequence. The second aim is to study tissue distribution and subcellular localization of NCAM containing the MSD. This is to understand the significance and mechanism of MSD function among NCAM variants. As a research model, we have chosen chicken heart development and focused on tissue differentiation in the myocardium in particular. We found that NCAM expression including species containing the MSD undergoes dramatic changes while myofibrillogenesis occurs. The present studies suggest the potential function of MSD-containing NCAM.

MATERIALS AND METHODS

Reagents

Reagents were purchased from the following sources: Keyhole limpet hemocyanin (KLH), Calbiochem (San Diego, CA); N-succinimidyl 3-(2-pyridyl dithio) propionate (SPDP) and BCA protein assay kit, Pierce (Rockford, IL); reagents for gel electrophoresis and Western blotting, Bio-Rad Laboratories (Hercules, CA); leupeptin, Boehringer Mannheim Corp. (Indianapolis, IN); neuraminidase from Streptococcus sp. (catalog number 151738), ICN Biomedical Inc. (Costa Mesa, CA); DE 52, Whatman Lab Sales Inc. (Hillsboro, OR); CNBr, Aldrich Chemical Co. Inc. (Milwaukee, WI). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) .

Antibodies

Anti-NCAM: NCAM from 14-day embryonic chicken brains, purified as described below or as previously described (32), was used to immunize rabbits. This antibody is frequently referred to as the pan NCAM antibody because it recognizes all forms of NCAM.

Anti-12AB and anti-12CD: Two peptides that together comprise the MSD region in chicken NCAM (Prediger et al., 1988) were synthesized by the MUSC Protein Sequence Facility. Oligopeptide 12AB contains the sequence encoded by exons 12A and 12B (single letter code RIPHSPSAAAPTCLAE); oligopeptide 12CD contains the sequence encoded by exons 12C and 12D (TTQPLPASESTEPPKG) (Figure 3). Each peptide was synthesized with two additional cysteine residues at its C-terminal. In order to induce strong immunogenicity, each oligopeptide was conjugated with KLH prior to immunization. First, Cys residues were reduced using 100 mM dithithreitol (DTT) for 30 min, and the peptides were desalted on G-25 Sephadex equilibrated in 0.1 M sodium phosphate/ 0.1 M sodium chloride (NaCl), pH 7.5. KLH was desalted using this same buffer, then activated with SPDP at a molar ratio of 1: 2,500 for 1 hour, then desalted again. Finally, the reduced oligopeptides were incubated with the activated KLH at a 500:1 molar ratio for 1 hour, then desalted. These oligopeptide-KLH conjugates were dialyzed against water, lyophilized, and resuspended in phosphate buffered saline (PBS). Subcutaneous inoculations into rabbits (50 μg NCAM or 1 mg oligopeptide-KLH conjugates per rabbit per injection) and IgG purification from serum were performed by standard methods (71).

Monoclonal antibodies 4d (specific for the ld form of NCAM) and V2E9 and JG22 (each specific for the β1 integrin subunit) were obtained from the Developmental Studies Hybridoma Bank. Monoclonal antibody NCD-2

(specific for N-cadherin) was a gift from Dr. Jack Lilien, Clemson University, Clemson, SC. Monoclonal antibodies against vinculin and α-actinin (sarcomeric) were obtained from Sigma Chemical Co. [Product Numbers V-9131 (clone hVIN-1) and A-7811 (clone EA-53) respectively].

Slot Blot

This simple assay allows the determination of the ability of an antibody to interact with a native (non-denatured) antigen. The antigen is placed in a plastic slot and transferred onto a nitrocellulose membrane by gravity or a mild vacuum (Slot Blot kit, Bio-Rad). The membrane-bound antigen is then detected by Western blotting. The membrane is blocked in tris buffered saline (TBS) containing 3 % gelatin, incubated with a primary antibody in the presence of 1% gelatin, incubated with a secondary antibody-alkaline phosphatase conjugate. Color development is performed in carbonate buffer, pH 9.8, using bromochloroindoyl phophate (BCIP) and nitroblue tetrazolium (NBT) substrates. The resulting color on a membrane is scanned using an Apple Macintosh computer and Adobe Photoshop software. The image information is then converted to numeric data using an Image Anaysis Program 1.4.

ELISA (Enzyme-Linked Immunoabsorbent Assay)

The sensitivity of this assay allows us an accurate estimation of the specific interaction occurring between antibody and antigen. 96-well microtiter plates are coated with various soluble antigens for 1 hr. Nonspecific protein binding sites are blolcked with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 2 hr. The primary antibody and then the secondary antibody, a HRP (horseradish peroxidase)-conjugate, are applied for 1 hr each in PBS containing 1% BSA. The color reaction is performed using substrates, 2, 2'-Azinobis (3-ethylbenzoothiazoline-6-sulfonic acid) diammonium salt (ABTS) and hydrogen peroxide (H_2O_2), and is quantitated by measuring the optical density (OD) at 414 nm using a plate reader.

Western Blot

Samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on minigels containing 6 % acrylamide except in the experiment on cyanogen bromide (CNBr) fragments of NCAM in which 12 % acrylamide was used and non-reducing conditions were used to enhance antigenicity. Proteins were transblotted to nitrocellulose using a semi-dry transfer method (Trans-Blot Semi-Dry Electrophoretic Transfer Cell, Bio-Rad). The nitrocellulose was blocked, incubated with primary antibodies, incubated with secondary antibodies (goat

anti-rabbit or anti-mouse IgGs conjugated to alkaline phosphatase), and color developed using BCIP and NBT as described as above (Reagents, Bio-Rad).

Preparation of Membranes/ NP40 Extraction/ Neuraminidase Treatment/ PIPLC Digestion

Purified membranes were prepared from 14-day embryonic chicken brains and 14-day embryonic and adult chicken hearts. Brains in cold PBS containing protease inhibitors (N-ethylmaleimide, 10 mM; benzamidine, 5 mM; leupeptin, 50 μg/ml; aprotinin, 2 μg/ml; phenylmethylsulfonylfluoride (PMSF), 2mM; pepstatin A, 5 μg/ml) were homogenized using a Braun-Sonic 2000 U ultrasonic generator. Homogenates were overlayed on PBS containing 42 % sucrose, then ultracentrifuged at 100,000 g for 30 min at 4° C. The membrane fraction which appeared above the 42 % sucrose was harvested, washed by dilution with cold PBS, and collected by centrifugation at 8 K rpm for 20 min in a JA10 rotor in a Beckman J2-HS High Speed Centrifuge. For heart membrane preparation, tissue was sonicated in ice-cold hypotonic buffer (200 mM sucrose/ 5 mM sodium phosphate, pH 7.5/ 1 mM (ethylene-dioxy) diethylenedinitro tetraacetic acid, EGTA) in the presence of protease inhibitors. Low-speed centrifugation, 300 g for 10 min, separated the membranes from nuclei and large debris. Membranes in the supernatant were then collected by ultracentrifugation at 100,000 g for 30 min.

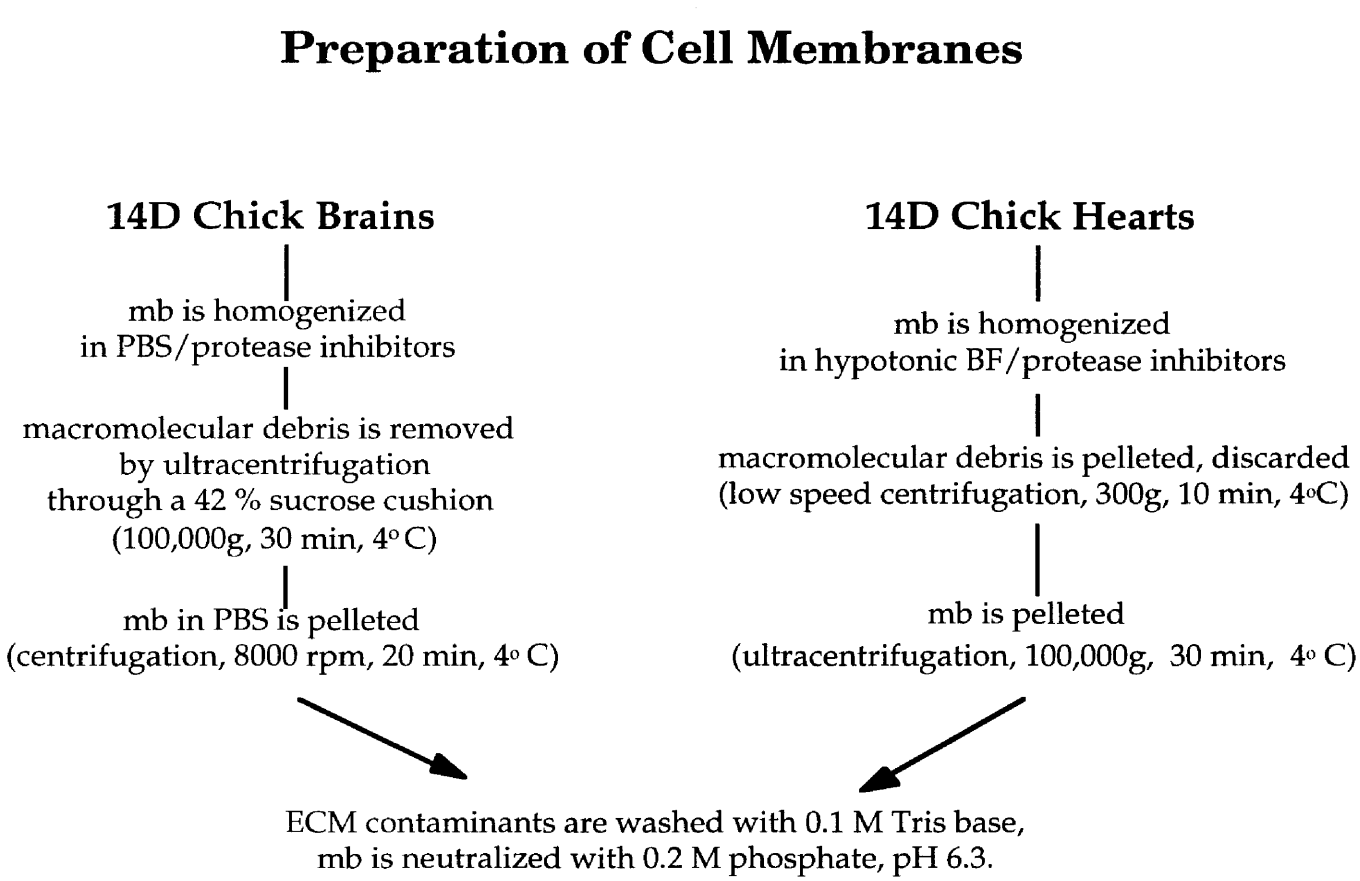

Figure 4. Preparation of Cell Membranes from Embryonic Chicken Tissue.

Embryonic day 14 chicken tissues, brain or heart, are isolated and homogenized. Cell membrane is fractionated from cytoplasmic contaminants by ultracentrifugation (see details in the Materials and Method section). Abbreviations: mb (membrane), BF (buffer), PBS (phosphate buffered saline), ECM (extracellular matrix)

Crude membranes were prepared from 4-day, 7-day, and 10-day embryonic chicken hearts and 5-day embryonic chicken ventricles and cushion tissue pads. Tissue in PBS was homogenized in the presence of protease inhibitors, then washed with PBS and harvested by two rounds of centrifugation (14,000 rpm, 3 min) in a microfuge (Figure 4).

To prepare nonidet P-40 (NP40) extracts for Western blotting and peanut lectin chromatrography, membranes were pelleted in a microfuge (14,000 rpm, 3 min), resuspended in 10 volumes of 0.5 % NP40/ PBS, and the extracts clarified in the microfuge (14,000 rpm, 5 min). The protein content of NP40 extracts was quantitated using the BCA assay.

To neuraminidase treat membranes, pelleted membranes were resuspended in 3 volumes of PBS containing 0.3 U/ ml of neuraminidase, incubated for 1 hour at 37° C, then washed with PBS and harvested by centrifugation. To neuraminidase treat NP40 extracts, neuraminidase was added to NP40 extracts to a final 0.3 U/ ml and incubated 1 hour, 37° C.

To phosphotidylinositol-specific phospholipase C (PIPLC) digest membranes, pelletted membranes were resuspended in 3 volumes of PBS containing 0.02 U/ ml of PIPLC (from Bacillus cereus, Sigma catalog number P 8804), incubated for 30 min at 37° C, and the PIPLC-released molecules in the supernatant harvested by centrifugation in a microfuge (14,000 rpm, 3 min). The residual membrane pellet was NP40 extracted as described above.

Immunoprecipitation of NCAM Molecules Containing the MSD Region

Purified membranes from 14-day embryonic chicken hearts (50 µl packed volume) were resuspended in 0.5 ml PBS containing 1mg/ml anti-12AB or anti-12CD immunoglobulin (IgG) and incubated for 1 hour at 4° C with agitation. Membranes were washed with PBS and harvested by two rounds of centrifugaton in a microfuge (14,000 rpm, 3 min). The membranes were extracted with NP40 as described above and antigen-antibody complexes were collected by incubation with 50 µl of Protein A-Sepharose for 1 hour at 4° C. The beads were then washed several times with 0.5 % NP40/ PBS. Bound NCAM was eluted by boiling the beads in SDS-PAGE sample buffer and Western blotted using the pan NCAM antibody as the primary antibody for detection. This procedure allowed immunoprecipitation experiments to be performed using anti-12AB and anti-12CD. Immunoprecipitation experiments performed with these antibodies in which the antibodies were added to proteins which had already been NP40 extracted gave uniformly negative results.

Peanut Lectin Chromatography

1.5 ml of NP40 extract from purified membranes from 14-day embryonic chicken hearts was incubated with 50 µl of peanut lectin-agarose (Sigma catalog number L 2507) for 1 hour at 4° C with agitation. The beads

were then washed several times with 0.5 % NP40/ PBS and bound proteins were eluted with 0.5 ml of 0.5 % NP40/ PBS/ 0.1 M galactose.

Purification of NCAM/ CNBr Digestion

Packed brain or heart membranes were resuspended in 10 volumes of 0.1 M Tris base to elute weakly attached proteins. Membranes were collected by ultracentrifugation and extracted in 10 volumes of 0.5 % NP40/ 0.2 M sodium phosphate, pH 6.3 containing protease inhibitors. Extracts were clarified by ultracentrifugation and applied to DE 52 equilibriated in 0.5 % NP-40/ 0.2 M sodium phosphate, pH 6.3 (10 ml of extract per ml of beads). The column was washed with 0.2 M sodium phosphate, pH 6.3 until no NP40 could be detected in the eluate. Contaminants were then eluted with 1 M NaCl/ 20 mM sodium phosphate, pH 6.5. The column was then washed with 25 mM octylthioglucopyranoside (OTG)/ 20 mM sodium phosphate, pH 6.5. Finally, NCAM was eluted from the DE52 using 0.55 M NaCl/ 25 mM OTG/ 20 mM sodium phosphate, pH 6.5. The eluate was dialyzed versus water and lyophilized. NCAM was further purified by HPLC gel filtration on a TosoHaas G4000SW column equilibrated with 25 mM OTG/20 mM sodium phosphate, pH 6.5 (Figure 5). This procedure yielded brain NCAM that was essentially 100 % pure as evaluated by SDS-PAGE and heart NCAM that was about 30 % pure.

Purification of NCAM

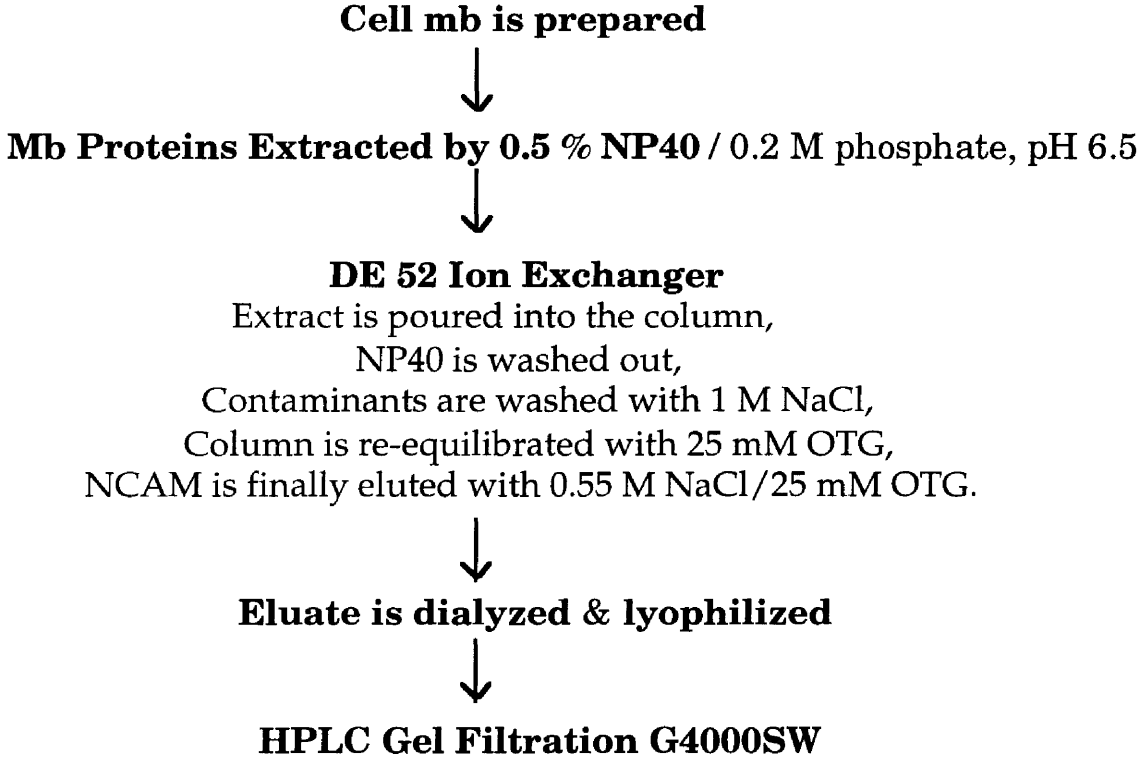

Figure 5. Purification of NCAM Molecules from Tissue Extract.

Proteins are extracted from the cell membrane fraction by NP40 (see Figure 4). Cell membrane extract is subjected to a DEAE anion exchange chromatography where the NCAM is eluted by a high salt buffer containing a detergent, OTG. NCAM is further purified by high pressure liquid chromatography using a G4000SW column. The purity of NCAM molecules is analyzed by gel electrophoresis and western blotting. Abbreviations: mb (membrane), NP40 (nonidet P-40), OTG (otyl thioglucopyranoside), HPLC (high pressue liquid chromatography).

Purified NCAM was dialyzed versus water, adjusted to a final 70 % formic acid, and treated with a large excess of CNBr under N_2 overnight, then dried in a Speed-Vac. This material was then dissolved in a small volume of 25 mM OTG/ PBS and neuraminidase treated as described above.

Immunohistochemistry

Hamburger-Hamilton (25) stage 18 chicken embryos were briefly rinsed in PBS and placed in plastic molds with Tissue-Tek compound media (ICN). Samples were quick frozen using liquid nitrogen. Five micron frozen sections were cut and mounted on poly-L-lysine-coated slide glasses. After rinsing off the embedding medium with distilled water and PBS, the sections were incubated at room temperature with 10 % normal goat serum /PBS for 30 minutes to block non-specific binding. Sections were incubated with primary antibodies (25 μg/ml for rabbit antibodies, 10 μg/ml for monoclonal antibodies except for anti-vinculin and anti-α-actinin which were used at 1:400 and 1:800 dilutions respectively as per the vendor's instructions) in 1% BSA/PBS in a humid chamber at 4 °C for 4-16 hours, then rinsed with 1% BSA/PBS. Appropriate fluorescein conjugated secondary antibodies [10 μg/ml of goat anti-rabbit IgG or goat anti-mouse IgG (Organon Teknika Corp., Durham, NC) in 1% BSA/PBS] were applied for one hour, followed by rinsing

and mounting in 90% glycerol, 0.2 M n-propyl gallate (22). Stained sections were examined by epifluorescent illumination using a Zeiss Axioscope.

Two experiments were performed to verify that the only molecules recognized by anti-12CD are NCAM molecules containing the peptide sequence used as immunogen. In one, the diluted antibody (25 μg/ml) was pre-absorbed by mixing with 1.25 μg/ml of the immunogenic peptide for 30 minutes at room temperature, followed by clarification at 13,000 g. In the second, tissue sections were preincubated with the pan NCAM antibody (25 μg/ml) for 4 hours prior to addition of anti-12CD (25 μg/ml). Both treatments completely blocked anti-12CD staining.

To examine the distribution of anti-12CD staining in cultured cells, cells were released from stage 18 hearts as described by Krug et al. (38) and depleted of fibroblasts as described by Lau (39). This method selectively removes the fibroblastic cells which grow rapidly in a monolayer culture than myocytes. Ventricle portions of hearts are trypsinized to disperse and the cell suspension is allowed to settle for 2 hours in a culture dish. Because fibroblasts adhere to the dish ahead within this period, the media mainly composed of myocytes is then transferred to a new culture plate. The enriched cardiomyocytes were plated on slides coated using a 20 μg/ml solution of fibronectin (New York Blood Center, New York, NY) and incubated for 3-7 days in the 'complete M199 medium' of Krug et al. (38). Cells were fixed with cold methanol (10 min, -20° C), then rinsed briefly with

50 % methanol/ 50 % PBS followed by a PBS rinse. Cells were stained as described above and examined by confocal microscopy.

Confocal Microscopy

As described above, 10-μm frozen sections were prepared and stained with anti-12CD as the primary antibody and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG as the secondary antibody. The sections were then double stained by further incubation with either monoclonal anti-α-actinin (sarcomeric) followed by tetramethylrhodamine isomer R (TRITC)-conjugated goat anti-mouse IgG or the pan NCAM antibody followed by TRITC-conjugated goat anti-rabbit IgG. Stained sections were examined by confocal microscopy using a Bio-Rad MRC-1000 equipped with a Zeiss Axioscope. The confocal iris was set at the smallest value to obtain the thinnest possible optical sections (< 0.25 μm).

RESULTS

Antibodies Specific to the MSD Region of NCAM

The whole NCAM gene is composed of 19 major exons. According to Nothern blot and cDNA sequence data, a subset of NCAM contains a peptide sequence that is encoded by four small alternatively spliced exons, 12A, B, C, and D (Figure 2). The small four exons are located between exons 12 and 13 of the NCAM gene. This portion of the molecule was originally (15) named the muscle specific domain (MSD); this nomenclature will be used here. The MSD found in the chicken heart consists of 31 amino acid. The localization of MSD is predicted to be between two fibronectin homologous repeats in the extracellular region of NCAM protein. The predicted localization and the deduced amino acid sequence of this region of NCAM are shown in Figure 2.

Since our initial goal was to identify the subset of heart NCAM that contains the MSD insert in its protein structure, it was necessary to produce an antibody specific to this MSD sequence. Using synthetic oligopeptides, two polyclonal antibodies were prepared against the MSD region of NCAM; one (anti-12AB) is against the polypeptide sequence encoded by exons 12A and 12B, the second (anti-12CD) against the polypeptide encoded by exons 12C and 12D (see Methods for details of antibody production). First, we tested the

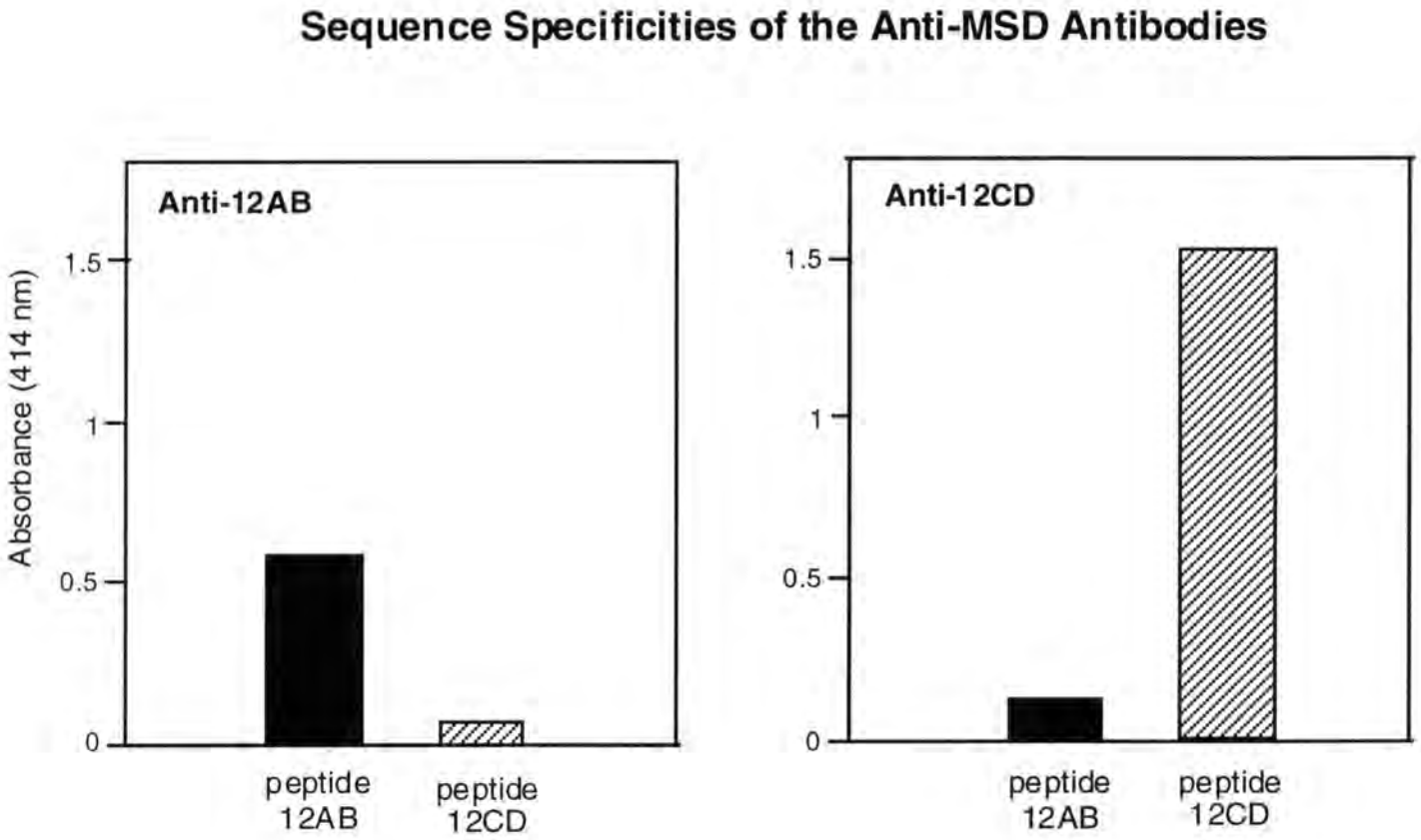

Figure 6. Specificity of Anti-MSD Antibodies against MSD Sequences.

MSD oligopeptides were challenged with anti-MSD antibodies in an ELISA. A 96-well microtiter plate was coated with 20 ng of 12AB or 12CD oligopeptide, incubated with the anti-MSD (anti-12AB or anti-12CD) antibody, then incubated with the secondary antibody-horseradish peroxidase conjugates. The subsequent color reaction measured at absorbance 414 nm is indicated at the y-axis with arbituary numbers. The results show that within the MSD sequence each anti-MSD recognizes an independent sequences; anti-12AB is specific to the 12AB sequence, whereas anti-12CD is specific to the 12CD sequence.

specificites of the two anti-MSD peptide antibodies by ELISA. Assay wells are coated with serial dilutions of MSD oligopeptide, the amount of anti-MSD bound to the peptide antigen is measured by peroxidase-mediated color reaction. Figure 6 demonstrates that the specificty of anti-12AB against the 12AB sequence as well as the specificity of anti-12CD against the 12CD sequence of MSD. Also, in the presence of competing peptide (the original immunogen), each anti-MSD antibody no longer recognizes its antigen (data not shown). The results consistently show that each anti-MSD recognizes the appropriate specific sequence within the MSD sequence.

Tissue-Specific Expression of the MSD Region in NCAM

Next, we wanted to examine the specificites of anti-MSD against the native NCAM molecules. We have used the slot blot method, which preserves native protein structure because the antigen is transferred to a membrane support without denaturation. NCAM was purified form 14-day embryonic brain or heart tissue extract as described in the Materials and Methods section. Corresponding slots in row A or B contain equal amounts of purified brain or heart NCAM, both of which are recognized by a pan anti-NCAM antibody (Figure 7). Various concentrations of purified brain or heart NCAM were incubated with an anti-MSD. While the pan anti-NCAM recognizes both brain and heart NCAM, the anti-12AB and anti-12CD recognize only heart NCAM.

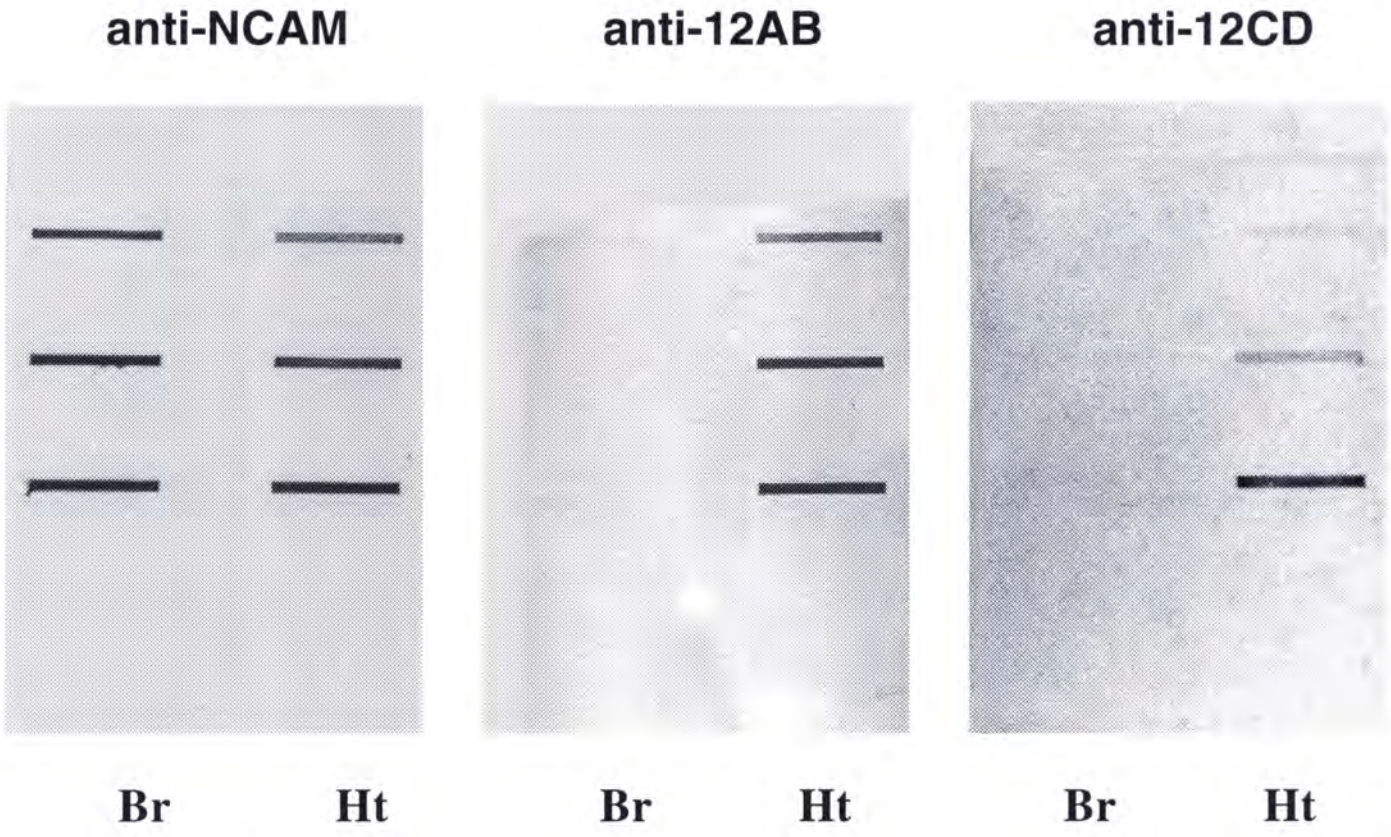

Figure 7. Anti-MSD Recognizes NCAM from Heart Tissue but Not from Brain.

The ability of anti-MSD to recognize intact NCAM molecules was examined by slot blotting. Matching slots in the left and right row contain equal amounts of purified brain and heart NCAM, respectively. From the top, slots contain 10 ng (top), 50 ng (middle), or 250 ng (bottom) of purified NCAM. The antigen immobilized in the slot was challenged with various anti-NCAM antibodies, then the result is shown by secondary antibody-mediated alkaline phosphatase color reaction. NCAM isolated either from brain or heart tissue is recognized by a pan anti-NCAM antibody. The anti-12AB or anti-12CD exclusively recognizes the NCAM species that are present in heart tissue.

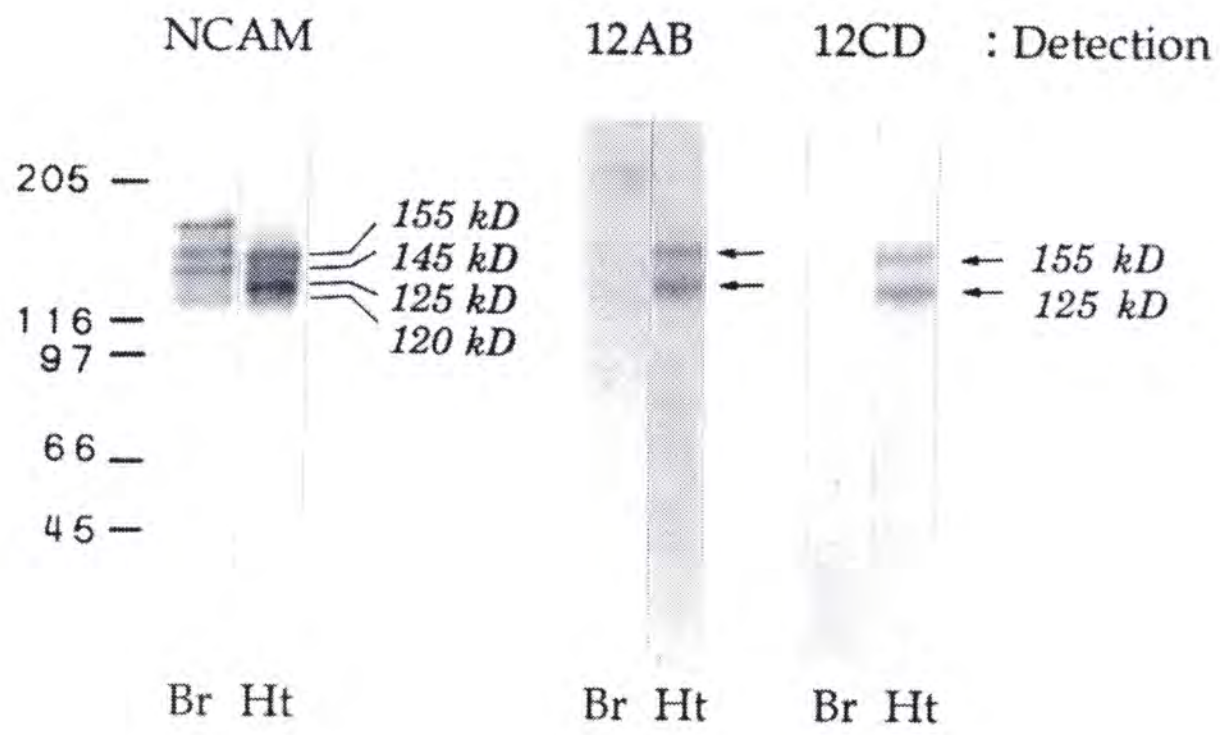

Figure 8. Specificity of Antibodies against the MSD Region of NCAM.

As described in the Methods, NP40 extracts of 14-day embryonic chicken heart and brain membranes were neuraminidase treated, then Western blotted using antibodies prepared against the indicated antigens as the primary antibodies for detection. Brain or heart extract is loaded to contain equal amount of NCAM to each lane; lanes containing heart extract (Ht) were loaded with 5 μg of protein for detection with the pan NCAM antibody and 15 μg of protein for detection with anti-12AB or anti-12CD, also, lanes containing brain extract (Br) were loaded with 1 μg of protein for detection with the pan NCAM antibody and 3 μg for detection with anti-12AB or anti-12CD. This 5:1 ratio of heart extract to brain extract results in similar levels of total NCAM detected with the pan NCAM antibody. The four forms of heart NCAM recognized by the pan NCAM antibody (155, 145, 125, and 120 kD) and the two forms of heart NCAM recognized by anti-12AB and anti-12CD (155 and 125 kD) are indicated. An extra band seen in the brain extract detected by anti-NCAM is the proteolytic fragment of NCAM produced during tissue preparation. The migration of standard proteins is indicated by their Mr x 10^{-3}.

Specificities of antibodies were further analyzed by Western blots. Crude membrane extract was prepared from the brain or heart tissue of 14-day chick embryo, then the ability of anti-MSD to recognize brain or heart NCAM was compared to that of antibody prepared against the entire NCAM molecule (Figure 8). Whereas the pan anti-NCAM recognizes all forms of NCAM expressed in the brain (180, 140, and 120 kD) or heart (155, 145, 125, and 120 kD), the anti-12AB and anti-12CD recognize only the heart NCAM. Among the NCAM species detected in heart, only two (155 and 125 kD) out of the four NCAM types are positive by anti-12AB or anti-12CD. Approximate molecular weights of four NCAM species detected in the heart are estimated as 155, 145, 125, and 120 kD. The results conclusively show that both anti-MSD antibodies specifically recognize the MSD sequence in NCAM. These results also indicate that the MSD region is specifically expressed in heart NCAM and that only subsets of heart NCAM species contain this MSD insert.

NCAM Splice Variants in Heart Tissue

ld, sd, and ssd Forms of NCAM All Can Contain the MSD Sequence

Three forms of NCAM are defined by their mode of attachment to the plasma membrane (9) (Figure 1). Two forms, ld and sd, are transmembrane proteins containing a large and small cytoplasmic domain, respectively. The third form, ssd, is GPI-linked to the plasma membrane. The ld NCAM has a

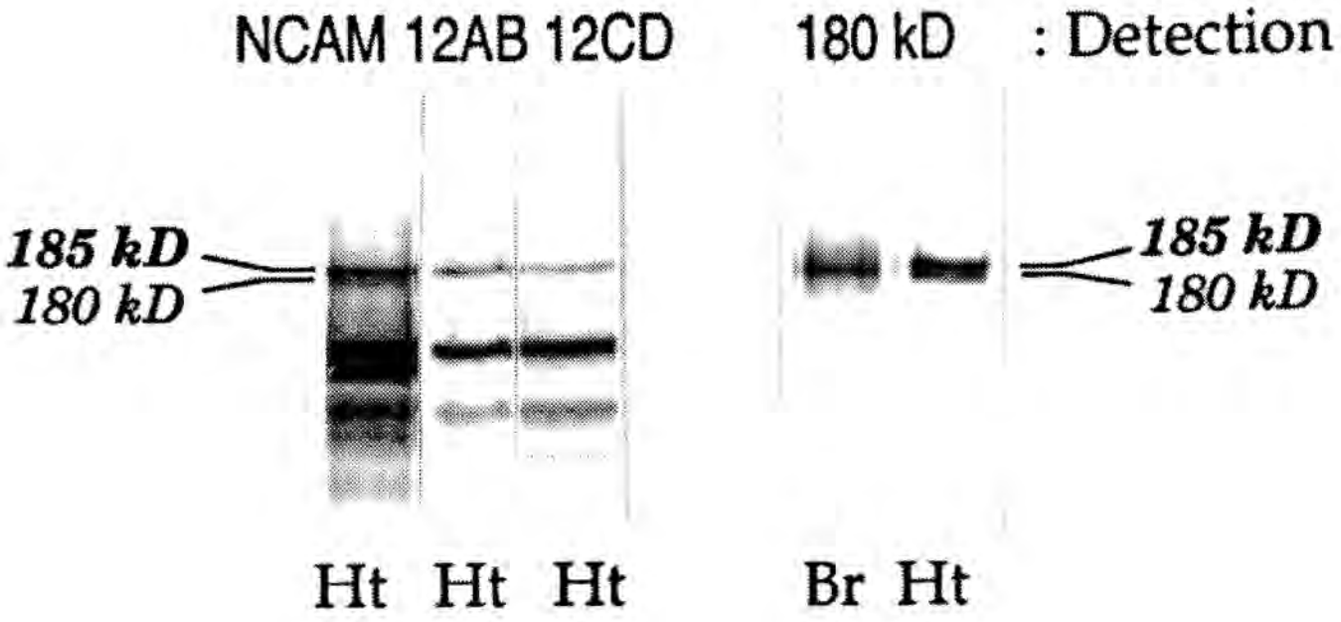

Figure 9. Forms of NCAM in 7-day Embryonic Heart Tissue.

As described in the Methods, NP40 extracts of membranes were neuraminidase treated, then Western blotted using antibodies specific for the indicated antigens as the primary antibodies for detection (180 kD indicates the use of monoclonal antibody 4d specific for the ld form of NCAM). Lanes containing 7-day embryonic heart extract (Ht) were loaded with 5 μg of protein for detection with the pan NCAM antibody or 4d and with 15 μg of protein for detection with anti-12AB or anti-12CD. The lane containing 14-day embryonic brain extract (Br) was loaded with 1 μg of protein. The 185 and 180 kD forms of heart NCAM recognized by a 4d (anti-180) antibody are indicated.

molecular weight of about 180 kD and had previously been demonstrated conclusively only in neural tissues.

Extract of 7-day embryonic hearts was analyzed for NCAM expression (Figure 9). Including the three basic forms of heart NCAM (the ld, sd, and ssd) that have been previously predicted by the molecular biology data, we detect total six different structural variants of NCAM from the day 7 embryonic heart. That is, the 185 and 180 kD forms are newly detected in addition to the four species (155, 145, 125, and 120 kD) observed in 14-day heart extracts (compare to Figure 8). The 185 kD, 145 kD, and 125 kD species all contain the MSD region which is detected by anti-12AB and anti-12CD (Figure 9).

To determine whether the 185 and 180 kD forms of NCAM observed in 7-day embryonic heart are authentic ld NCAM, extracts were Western blotted using monoclonal antibody 4d specific for this form of NCAM (76) (Figure 9). Both the 185 and 180 kD forms of heart NCAM, as well as a form of brain NCAM of similar molecular mass (known as the 180 kD), were recognized by this anti-180 antibody. These results indicate that two forms of ld NCAM (185 and 180 kD) are present in heart extracts, and that one (185 kD) of those ld forms also contains the MSD region.

Transmembrane *versus* GPI-linked Forms of NCAM

To determine which of the forms of NCAM we observe are GPI-linked to the plasma membrane, membranes from 14-day embryonic heart tissue

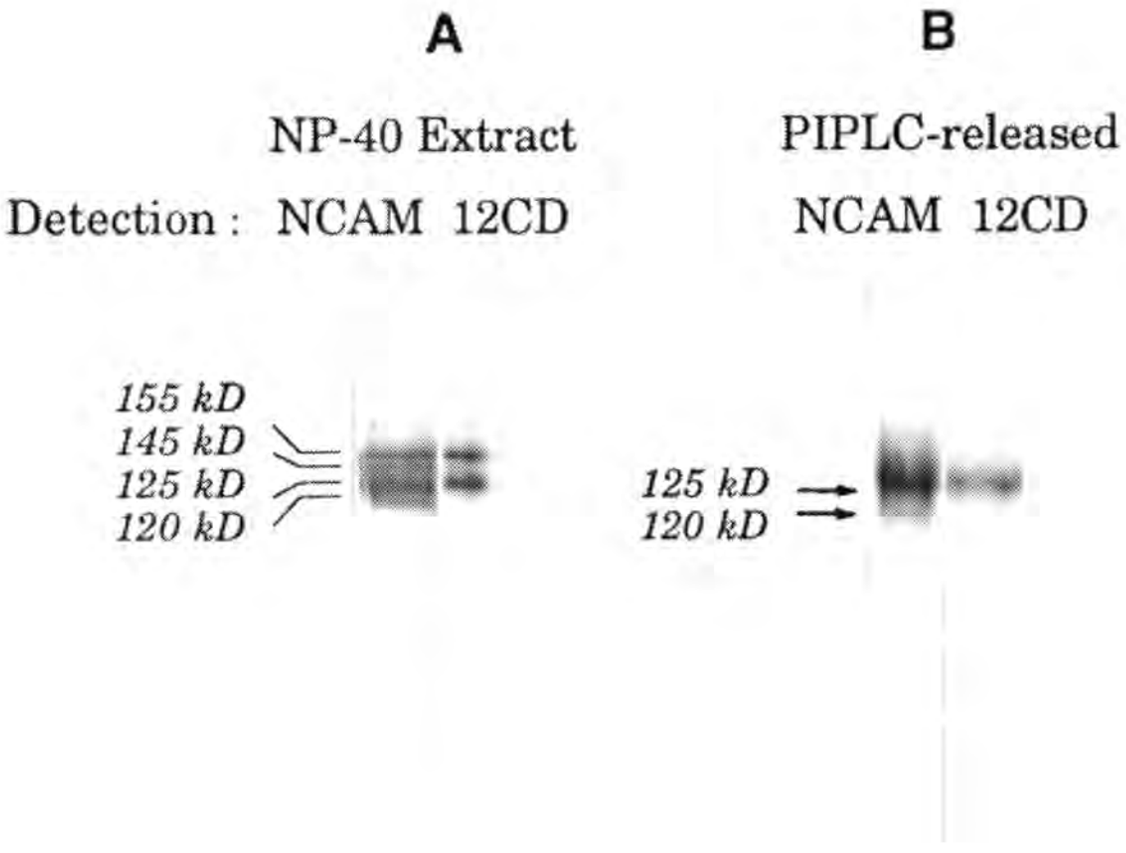

Figure 10. Transmembrane and GPI-linked Forms of NCAM.

As described in the Methods, 14-day embryonic heart membranes were neuraminidase treated, treated with PIPLC to release GPI-linked proteins, and the residual membranes were extracted with NP40. NP40 extracts (5 μg for detection with the pan NCAM antibody; 15 μg for detection with anti-12CD) and PIPLC-released material (from equivalent amounts of membranes as the NP40 extracts) were Western blotted using antibodies prepared against the indicated antigens as the primary antigens for detection. The four forms (recognized by the pan NCAM antibody) of heart NCAM in NP40 extracts (155, 145, 125, and 120 kD) and the two PIPLC-releasable forms of heart NCAM (125 and 120 kD) are indicated.

NCAM Splice Variants in Brain or Heart Tissue

mode of mb attachment	Brain	Heart
ld	180 kD	**185 kD** (MSD+, O-linked Sugar+ ?) 180 kD
sd	140 kD	**155 kD** (MSD+, O-linked Sugar+) 145 kD
ssd	120 kD	**125 kD** (MSD+, O-linked Sugar+) 120 kD

Table 1. Comparison of the Forms of NCAM in Brain and Heart Tissues.

NCAM consists of three structural forms defined by their modes of membrane attachment. In heart, these ld, sd, or ssd forms are expressed with or without the MSD sequence, whereas no MSD is expressed in brain NCAM. A notable feature of the MSD is that it contains serine and threonine sequences which are acceptor sites for O-linked oligosaccharides as demonstrated by peanut lectin binding specificity (Figure 13). We assume the 185 kD form also binds to the peanut lectin as the other MSD containing NCAM species (145 kD and 125 kD) do.

were treated with PIPLC to release the GPI-linked forms. NP-40 extracts of the residual membranes (Figure 10, A) or the PIPLC-released material (Figure 10, B) were then Western blotted with either anti-NCAM or anti-12CD. Only the 125 and 120 kD forms of NCAM were released by PIPLC, and of these, only the 125 kD form was recognized by anti-12CD. As in Figure 8, the 155, 145, 125, and 120 kD forms of NCAM were present in the residual membranes, of which only the 155 and 125 kD forms were recognized by anti-12CD. Thus, it appears that the 185 and 180 kD forms of NCAM are ld, the 155 and 145 kD forms are sd, and the 125 and 120 kD forms are ssd. In each pair, there is a higher molecular weight species containing the MSD region and a lower molecular weight species lacking the MSD region (summarized in Table 1).

The MSD Region Does Not Show Heterogeneity In Molecular Weight

PCR amplification studies of rat heart cDNAs suggested that alternative splicing occurs among exons 12A-D which should lead to multiple forms of NCAM with MSD regions of different sizes (Reyes et al., 1991). In contrast, our results show no evidence for size heterogeneity in the MSD regions of ld, sd, or ssd NCAM from either 7-day or 14-day embryonic hearts (Figures 8, 9, and 10). To evaluate whether we do not observe size heterogeneity because the differences in the sizes of the variants are too small compared to the size of the intact NCAM molecule, CNBr fragments of heart NCAM were prepared and Western blotted with anti-NCAM, anti-12AB, and anti-12CD

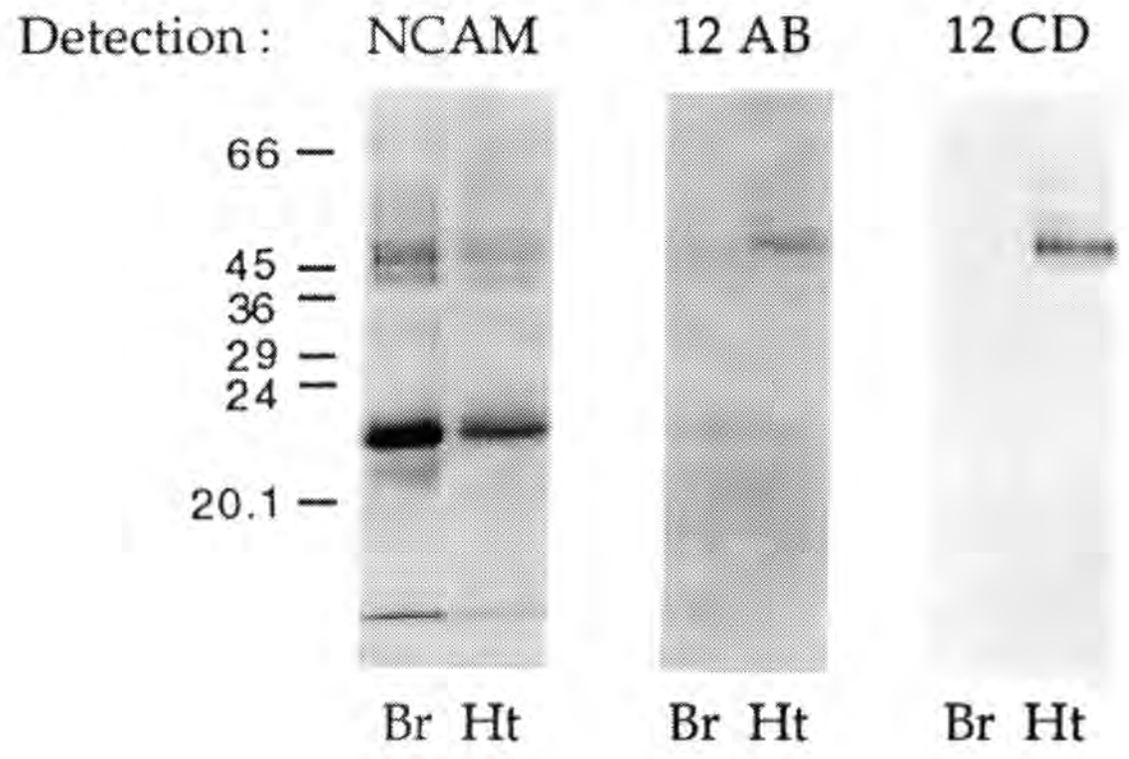

Figure 11. MSD region in CNBr digests of NCAM.

As described in the Methods, NCAM was purified from 14-day embryonic brain and heart membranes, digested with CNBr, neuraminidase treated, and Western blotted using antibodies prepared against the indicated antigens as the primary antibodies for detection. Lanes containing brain NCAM CNBr fragments (Br) were loaded with 10 μg of protein for detection with the pan NCAM antibody and 30 μg of protein for detection with anti-12AB or anti-12CD; lanes containing heart NCAM CNBr fragments (Ht) were loaded with 30 μg of protein for detection with the pan NCAM antibody and 90 μg of protein for detection with anti-12AB or anti-12CD. This ratio of brain and heart NCAM CNBr fragments was chosen to provide similar levels of signal using the pan NCAM antibody for detection. The migration of standard proteins is indicated by their Mr x 10^{-3}.

(Figure 11). As expected from the localization of the MSD region within NCAM (Dickson et al., 1987), anti-12AB and anti-12CD recognized a single CNBr fragment of about 45 kD. No size heterogeneity in the MSD region was seen even in these smaller CNBr digests. In control experiments, no CNBr fragments of brain NCAM were recognized by either anti-12AB or anti-12CD, even though the profiles of CNBr fragments of brain and heart NCAM recognized by anti-NCAM were similar. These results indicate that, at the protein level, only one form of the MSD region can be detected in our extracts, presumably that encoded by mRNAs containing the transcripts from all of exons 12A, 12B, 12C, and 12D.

Polysialic Acid on NCAM Inhibits the Anti-MSD Accessibility to the Molecule

NCAM molecules bear unique, massive polysialic acid-containing oligosaccharide(s) in a close proximity to the MSD region (9) (Figure 1). To determine whether this structure interferes with the ability of anti-MSD region antibodies to bind to the molecule, the ability of anti-12AB and anti-12CD to recognize NCAM molecules before and after neuraminidase treatment was compared (Figure 12). Whereas polysialic acid does not interfere with the ability of anti-NCAM to recognize NCAM, anti-12AB and anti-12CD do not recognize NCAM in Western blots (Figure 12, A) or by immunoprecipitation (Figure 12, B) unless the polysialic acid is first removed

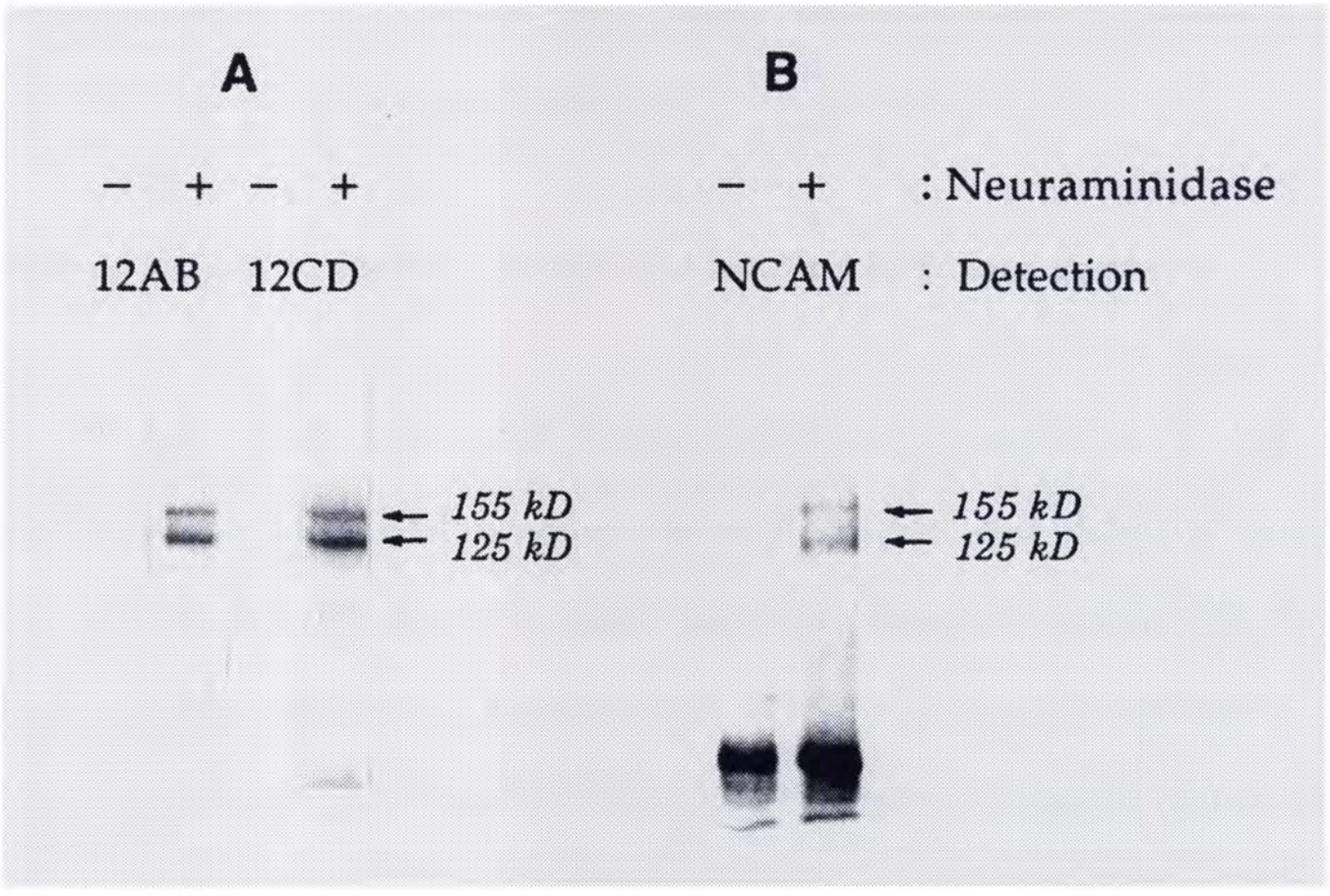

Figure 12. Effect of Polysialic Acid on the Recognition of NCAM by Anti-12AB and Anti-12CD.

A) Western blots. As described in the Methods, NP40 extracts of 14-day embryonic heart membranes (15 μg aliquots) either without (-) or with (+) neuraminidase treatment were Western blotted using antibodies against the indicated antigens as the primary antibodies for detection. B) Immunoprecipitation. As described in the Methods, NCAM from 14-day embryonic heart plasma membranes, either without (-) or with (+) neuraminidase treatment, was immunoprecipitated using anti-12CD IgG. This material was Western blotted using the pan NCAM antibody as the primary antibody for detection. Similar results were obtained in immunoprecipitation experiments using anti-12AB IgG (data not shown). The heavy band near the bottom of both lanes represents IgG heavy chain. The migration of the 155 and 125 kD forms of NCAM containing the MSD region is shown in both A and B.

from the molecule. In contrast to the Western blot, anti-12CD does recognize NCAM equally well before and after neuraminidase treatment in immunohistochemical assays (Figure 16, 17, 18, 19, 20, and 22).

Forms of NCAM Containing the MSD Also Bind to Peanut Lectin

NCAM from a skeletal muscle cell line consists of a 155 kD transmembrane form that contains the MSD region and binds to peanut lectin as well as a 125 kD GPI-linked form that does not contain the MSD region and does not bind to peanut lectin (75). In order to characterize the peanut lectin binding of heart NCAM, NP40 extracts of plasma membranes from 14-day embryonic heart tissue were fractionated on immobilized peanut lectin; the forms of NCAM in the total and eluate were then detected by Western blotting with anti-NCAM, anti-12AB and 12CD (Figure 13). NCAM molecules bind to peanut lectin only after neuraminidase treatment; even then, only the two polypeptides (155 and 125 kD) recognized by anti-12AB and anti-12CD bind to peanut lectin. These results are consistent with the facts that peanut-lectin binding oligosaccharides are believed to be O-linked and that the MSD region contains two putative acceptor sites composed of Ser or Thr residues for O-glycosylation.

Developmental Changes in the Expression of NCAM Splice Variants

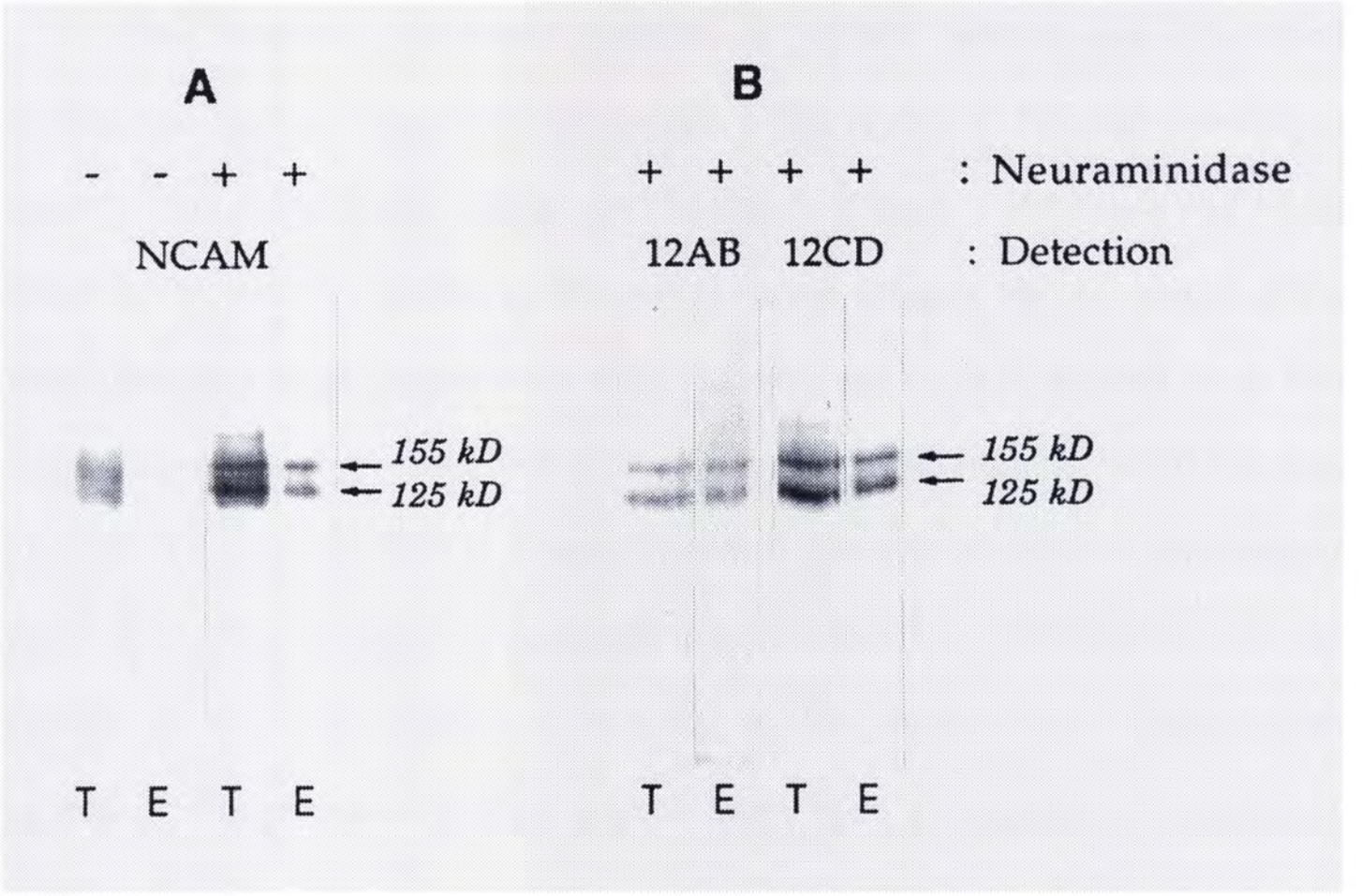

Figure 13. Binding of NCAM to Immobilized Peanut Lectin.

As described in the Methods, NP40 extracts of 14-day embryonic heart membranes, either untreated (-) or treated (+) with neuraminindase, were incubated with peanut lectin-agarose. Aliquots of the total extract (T) and the 0.1 M galactose eluate (E) were Western blotted using antibodies against the indicated antigens as the primary antibodies for detection. Lanes containing total extract were loaded with 5 μg of protein for detection with the pan NCAM antibody and with 15 μg of protein for detection with anti-12AB or anti-12CD; lanes containing the galactose eluate were loaded with the material purified from 20 μg of extract for detection with the pan NCAM antibody and with the material purified from 60 μg of extract for detection with anti-12AB or anti-12CD. The migration of the 155 and 125 kD forms of NCAM that are specifically precipitated by immobilized peanut lectin and are specifically recognized by anti-MSD region antibodies are indicated.

The temporal expression pattern of NCAM species was examined during the course of heart development. The forms of NCAM present in extracts from 4-day, 7-day, 10-day, and 14-day embryonic heart tissue and adult heart tissue were compared by Western blotting (Figure 14). Dramatic shifts were observed in the expression of ld, sd, and ssd NCAM as well as in the proportions of each of these forms containing the MSD region. ld NCAM was present at 4 days, peaked at 7 days, was still present at 10 days, but absent thereafter. This observation supports our idea that the ld NCAM is actually present in the heart (Figure 9) and not in the innervation of the heart, because a different time course would have been expected for a molecule associated with the innervation of the heart (peaking later and more persistent) (77). The sd form of NCAM is expressed in a similar time course as ld, while the ssd is expressed later in development and is essentially the only form observed in adult tissue.

The ratio of MSD positive to MSD negative forms of NCAM varies during development. For ld, sd, and ssd NCAM the percentage of the molecules containing the MSD region always increases during development (Figure 14). For example, in 7-day tissue the MSD-positive 185 and 155 kD forms of ld and sd NCAM are present in similar concentrations to the MSD-negative 180 and 145 kD forms of ld and sd. By 10 days, these MSD-positive forms are clearly present at higher concentrations than the corresponding MSD-negative forms. Similarly, the 120 kD MSD-negative form of ssd

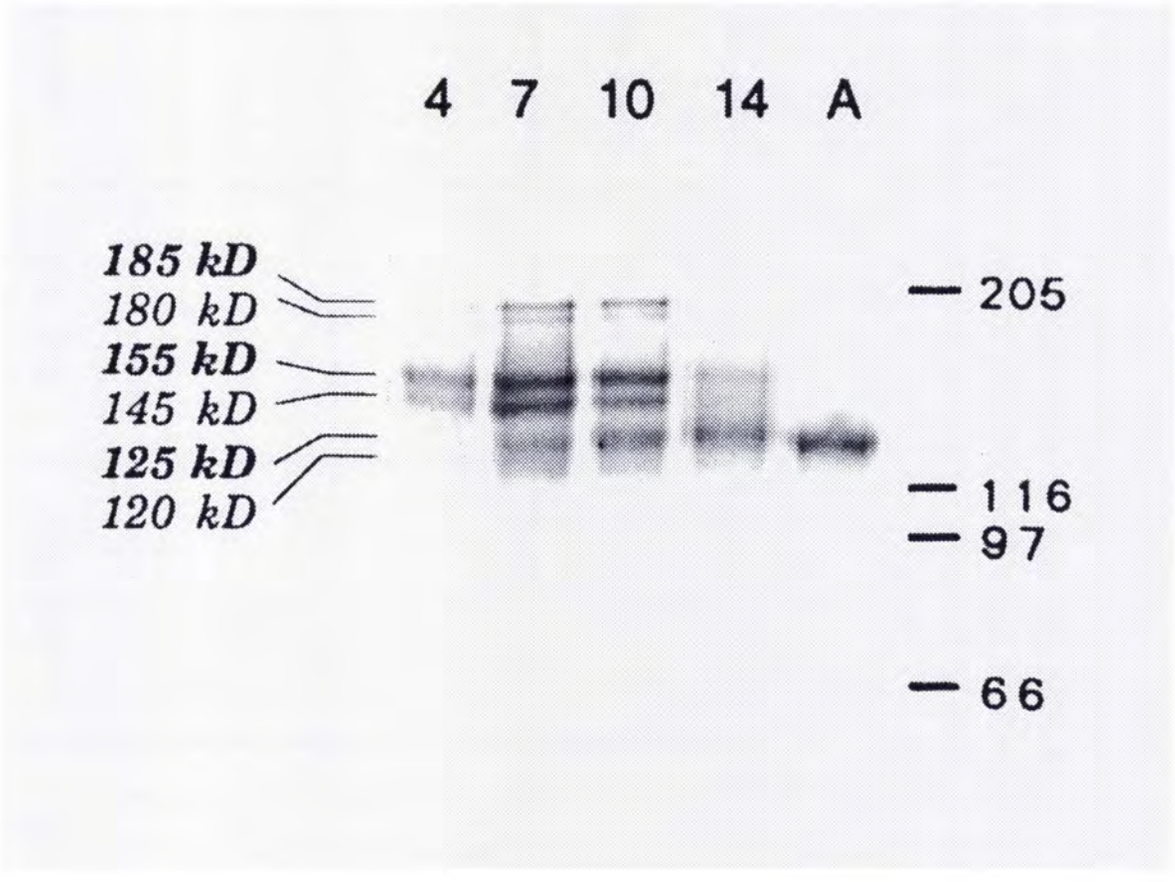

Figure 14. Forms of NCAM Present in the Developing Heart.

As described in the Methods, 5 μg aliquots of NP40 extracts of membranes from 4-day (4), 7-day (7), 10-day (10), and 14-day (14) embryonic heart tissue, and from adult (A) heart tissue were neuraminidase treated, then Western blotted using the pan NCAM antibody as the primary antibody for detection. The migrations of the 185, 180, 155, 145, 125, and 120 kD forms of NCAM are indicated; those containing the MSD region (185, 155, and 125 kD) are in bold. The identity of these forms of NCAM was confirmed in parallel experiments using anti-12AB and anti-12CD for detection (data not shown). The migration of standard proteins is indicated by their Mr x 10^{-3}.

Developmental Changes in Expression of theNCAM Splice Variants

mb attachment	MW	day 4	day 7	day 10	day 14	adult
ld	185 kD (MSD+) 180 kD	+ +	++ +	++ +		
sd	155 kD (MSD+) 145 kD	++++ +++	++++ ++++	++++ ++	++ +	
ssd	125 kD (MSD+) 120 kD	+ +	++ +	++ +	++++ +	++++++++

Table 2. Developmental Changes in NCAM Species.

Chicken hearts are isolated from the different stages of development and extracted with NP40. When similar amounts of tissue extract are loaded to the gel, we observe that the ratios of NCAM species changes dramatically according to heart development (Figure 14). Whereas the the membrane bound forms of NCAM (ld. sd) are abundantly expressed at earlier stages, the PIPLC-linked forms (ssd) are dominant at later stages. In adult, most NCAM are expressed as the PIPLC-releasable form. At any time of development, the MSD containing NCAM subspecies compose a bigger population than the ones lacking it. Also, the percentage of the MSD-positive NCAM increases as development proceeds.

NCAM is present in 7-day tissue but below detection thereafter, while the 125 kD MSD-positive form of ssd NCAM continues at high concentration throughout development (summarized in Table 2).

Spatial Variation in the Expression of NCAM Species

In order to understand the role of NCAM species during heart morphogenesis, we have carefully examined the tissue distribution and subcellular localization of various NCAM forms. Spatial variations in the expression of forms of NCAM are readily detected in tissue extract prepared from different compartments of heart. Ventricular tissue (primarily myocardium) from 5-day embryos contains a similar set of forms of NCAM (Figure 15) to that observed in the 4-day or 7-day heart (Figure 14). In contrast, atrioventricular cushion tissue from 5-day embryos contains primarily one form of NCAM, the MSD-negative form of sd. It contains much less ld, ssd, or even the MSD-positive form of sd NCAM than does the ventricular extract from the same stage embryos. Moreover, the small amount of MSD-positive sd present may be due to myocardial contamination of the cushion tissue given that cultured endocardial or mesenchymal cells contain only one form of NCAM, MSD-negative sd (Mironov, V., R. R. Markwald, and S. Hoffman, unpublished observations). Thus, the percentage of MSD-positive NCAM in heart tissue can vary from almost 0 to 100 % depending on the stage of development and region of the heart.

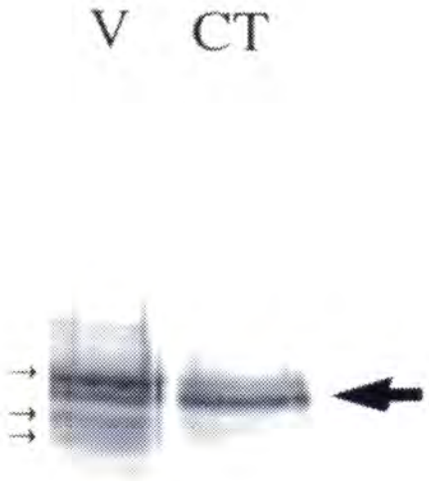

Figure 15. Endocardial Cushion Tissue is Deficient in GPI-linked and MSD Region-containing Forms of NCAM.

Ventricles (V) and cushion tissue (CT) pads were dissected from five-day chicken embryos. As described in the Methods, 5 μg aliquots of NP40 extracts of membranes were neuraminidase treated, then Western blotted using the pan NCAM antibody as the primary antibody for detection. Small arrows on the left indicate the forms of NCAM (155, 125, and 120 kD) present at relatively high levels in the ventricle and low levels in cushion tissue; the large arrow on the right indicates the form of N-CAM (145 kD) present at relatively high levels in both samples.

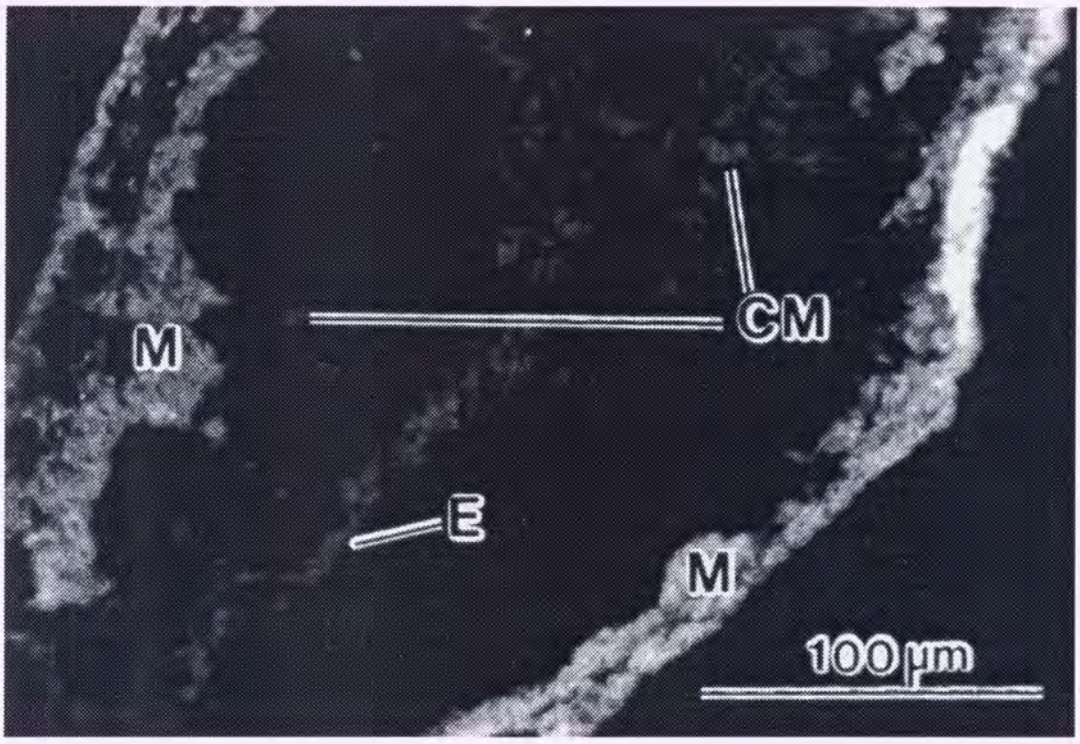

Figure 16. Anti-12CD immunostaining of the Stage 18 Chicken Heart.

Anti-12CD immunostaining was detected in the myocardium (M), whereas little staining was revealed in either endocardium (E) or in cushion mesenchyme (CM). Scale bar = 100 µm.

In order to determine the distribution at the immunohistochemical level of NCAM molecules containing the MSD region, sections of stage 18 embryonic heart tissue were stained using anti-12CD (Figure 16). Since anti-12AB was not useful in immunohistochemical studies, detection of the MSD in tissues was mainly dependent on using anti-12CD. As predicted from the results of Figure 14, the myocardium was stained with anti-12CD, while little or no staining was seen in the endocardium or endocardial cushion tissue.

When the myocardial portion of these sections was observed at high magnification, a striking subcellular distribution of NCAM molecules containing the MSD region was observed; staining with anti-12CD appeared to coincide with the position of Z discs (Figure 17). This staining was specific; no staining was observed if the antibody was pre-incubated with the peptide used to produce anti-12CD (Figure 17, b) and no staining was observed with non-immune IgG (Figure 17, f). Although anti-NCAM antibodies did stain the surface of myocardial cells, they did not highlight Z discs (Figure 17, c). Other adhesion molecules, N-cadherin and β1 integrin, have previously been shown to be associated with Z discs later in development (29, 37, 68). We examined the expression of these proteins in stage 18 myocardium; while both molecules were present, neither N-cadherin (Figure 17, d) nor β1 integrin (Figure 17, e) colocalized with Z discs. Therefore, at this early stage in development, NCAM molecules containing the MSD region appear to

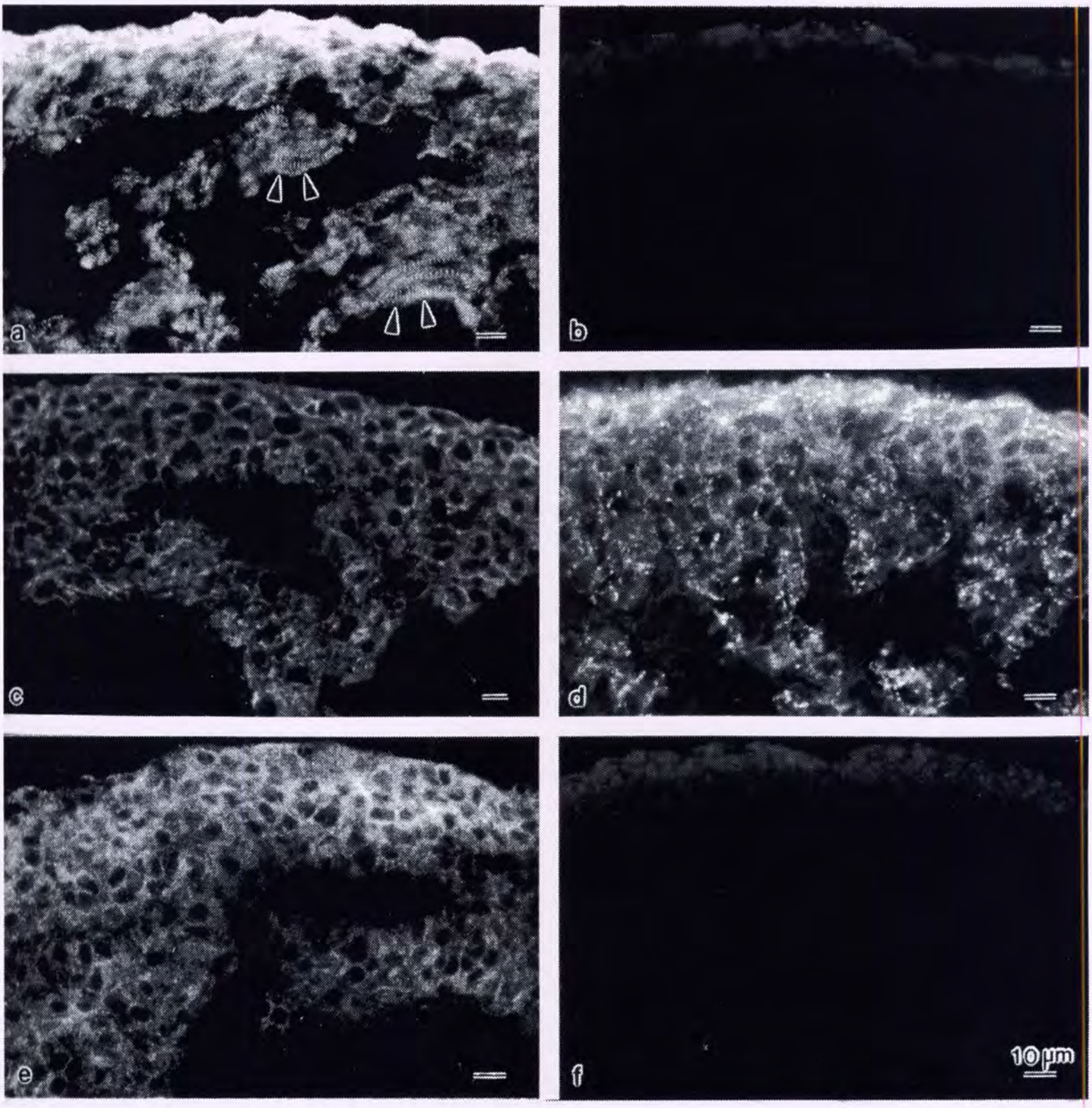
a
b
c
d
e
f
10 µm

Figure 17. Comparative Immunostaining of Different Adhesion Molecules in the Stage 18 Chicken Heart Myocardium.

a) High magnification photomicrograph depicts a striking subcellular distribution of anti-12CD staining (arrowheads) that coincides with the position of Z discs. b) A section adjacent to that shown in a) in which anti-12CD was pre-absorbed with the immunogenic peptide as described in the Methods. Note that this treatment prevented the immunostaining seen in a). c) Immunostaining with the pan NCAM antibody was localized at the periphery of the myocardial cells. d) Anti-N-cadherin (NCD-2) immunostaining was found in a punctate pattern at the periphery of the myocardial cells. e) Anti-β1 integrin (V2E9) immunostaining labeled the cell periphery. Similar results were obtained with anti-β1 integrin antibody JG22. f) No staining was observed with non-immune rabbit IgG. Scale bar = 10 μm.

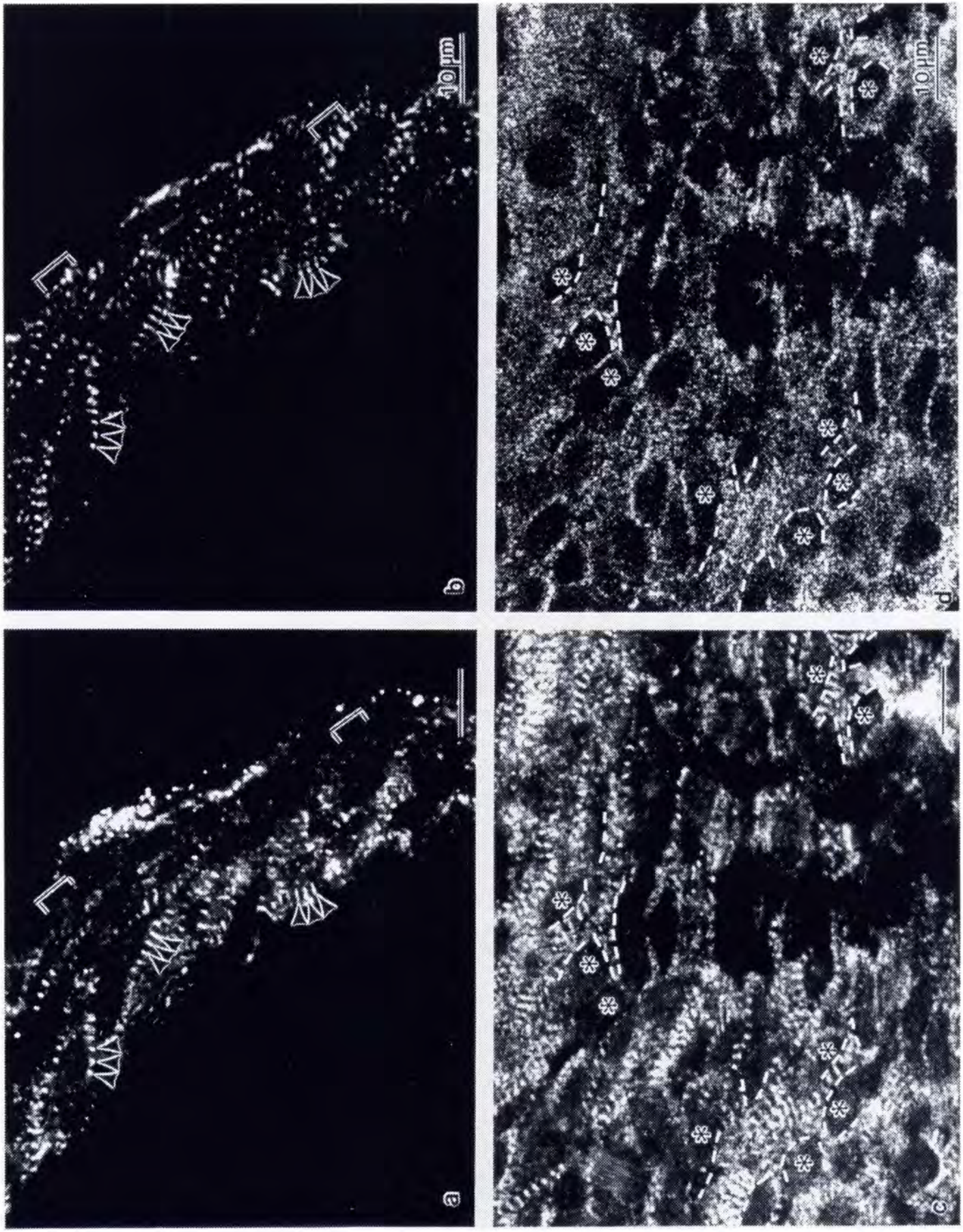
10 μm
b
10 μm
d
a
c

Figure 18. Confocal Microscopic Observation of Stage 18 Chicken Heart Doubly Stained with Anti-12CD and Anti-α-Actinin (sarcomeric) or Anti-12CD and Anti-NCAM.

Double immunostaining routinely reveals a coincidence between anti-12CD (arrowheads in a) and α-actinin (arrowheads in b) staining in association with Z discs. Occasionally anti-a-actinin staining is observed in the absence of anti-12CD staining (compare brackets in a and b). In contrast, there is only limited overlap between anti-12CD staining (c) and staining with the pan NCAM antibody (d). Cells in which the plane of focus captured the cell surface (regions between broken lines) showed almost uniform staining with the pan NCAM antibody (d) whereas anti-12CD staining appeared in the characteristic pattern (c). Cells cut in equatorial section (asterisks inside) showed almost uniform circumferential staining with the pan NCAM antibody (d) and frequently show periodic circumferential staining with anti-12CD (c). Scale bar = 10 μm.

colocalize with Z discs, while N-cadherin, β1 integrin, and NCAM molecules lacking the MSD region do not exhibit this specific association.

Confocal microscopy was used to confirm the localization of Z discs and forms of NCAM. When tissue sections were double-labeled using anti-12CD and anti-α-actinin (an actin binding protein which is a marker for Z discs), an almost complete coincidence between the two signals was observed (compare Figures 18, a and b). In two regions (indicated by brackets), periodic anti-α–actinin staining was observed, but no staining with anti-12CD. These observations suggest that most Z discs in these cells are located close to the cell surface in the same confocal section (thickness is about 2 μm) as the MSD region-containing NCAM on the cell surface. Only the occasional set of Z discs is located either deeper in the cell, or is not associated with NCAM molecules containing the MSD region.

When tissue sections were double-labeled using anti-12CD and the pan NCAM antibody, cells in which the plane of focus captured the cell surface showed almost uniform staining with the pan NCAM antibody, whereas anti-12CD staining appeared in the characteristic striped pattern (compare Figures 18, c and d, regions between broken lines). Only at scattered sites did the pan-NCAM antibody show any hint of highlighting NCAM molecules associated with Z discs. Similarly, cells in which the plane of focus cut an equatorial section (Figures 18, c and d, asterisks inside) showed circumferential staining which was relatively uniform with the pan NCAM

antibody and which was frequently periodic with anti-12CD. These results strongly suggest that NCAM molecules containing the MSD region are colocalized with Z discs while NCAM molecules lacking the MSD are uniformly distributed over the entire cell surface.

Despite the fact that the anti-12CD antibody recognizes only NCAM in Western blotting experiments, we were concerned that it might recognize an additional protein in immunohistochemical studies. To rule out this possibility, tissue sections were sequentially incubated with the pan NCAM antibody and anti-12CD antibodies. This pretreatment with the pan NCAM antibody totally blocked anti-12CD staining (data not shown) indicating that the pan NCAM antibody and anti-12CD recognize the same protein, i.e. NCAM, and that anti-12CD does not recognize non-NCAM epitope.

Structures known as 'costameres' have been identified by Pardo et al. (1983). A costamere is defined as a structure where the cell surface proteins are linked to subcelluar Z discs by a vinculin-containing structure. When we examined the distribution of vinculin in tissue sections from stage 18 myocardium, however, the staining patterns observed did not coincide with the patterns obtained with anti-12CD or anti-α-actinin (Figure 18). Additional data (Figure 22) indicates that the association of vinculin with Z discs (costamere assembly) has not yet occurred at stage 18.

Subcellular Localization of NCAM Splice Variants

in Cultured Carodiomyocytes

Subcellular distribution of MSD containing NCAM species was also examined in stage 18 cultured cardiomyocytes. We have established a primary culture system that consists of over 90 % homogenous cardiomyocyte cells (see the Materials and Methods section). Dispersed myocytes show primitive muscle contraction upon attachment to the culture dish. After one or two days in culture, myocytes organize their arrays of intracellular myofibrils (shown in Figure 19). As the culture proceeds, cells form a web-like monolayer between adjacent cells via extended myopodia. Muscle contraction is regular, rhythmic, and coordinated at this stage of culture, which indicates completion of myofiber assembly in the cell. This in vitro property of myofiber disassembly-and-reassembly in culture much resembles the in vivo embryological myofibrillogenesis that occurs during mucle tissue differentiation (58).

When NCAM was examined in cultured stage 18 myocardial cells, anti-12CD staining was in register with Z discs (Figure 19, a). Similar localization is also observed in vivo, in cardiomyocyte tissue of the developing heart (Figure 17 and 18); the double immunostaining result shows that the MSD-containing NCAM found at Z-discs are colocalized with α-sarcomeric actinin (compare Figure 18, a and b). The 12CD staining pattern also depicts the rib-like stripes at the cell surface of cultured cardiomyocytes.

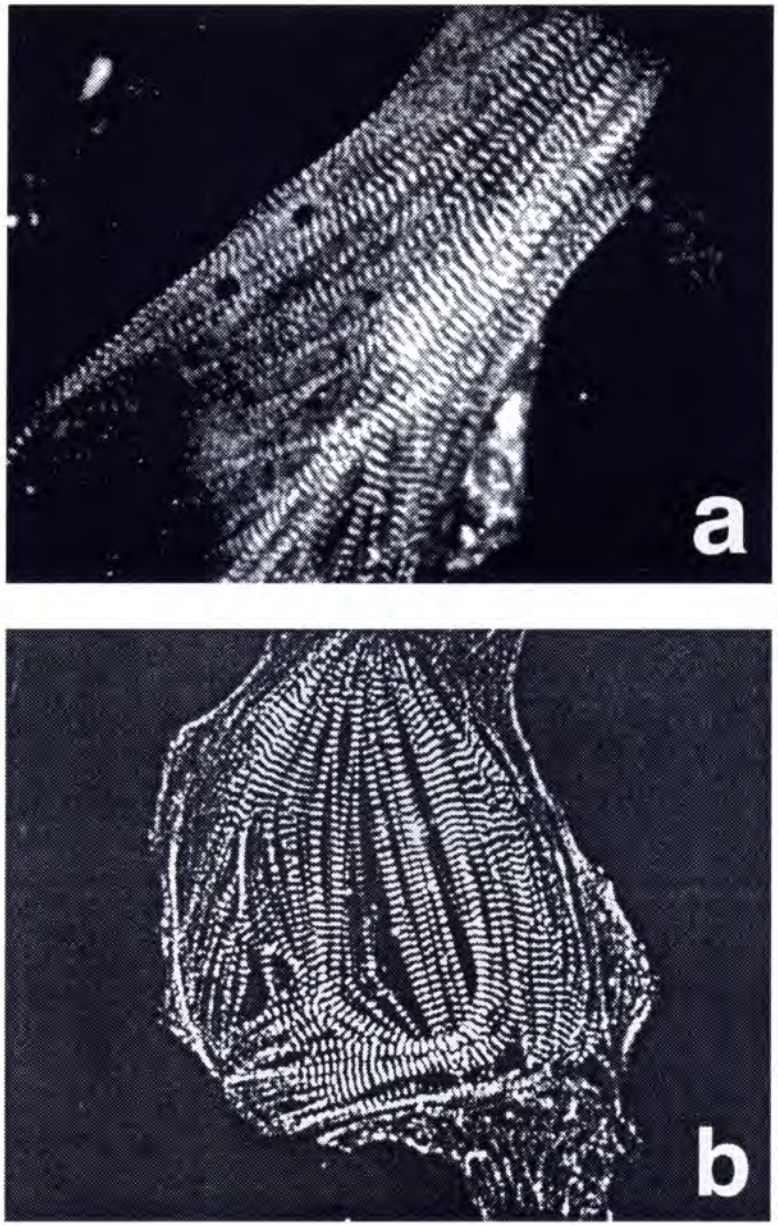

Figure 19. Confocal Microscopic Observation of the Cultured Stage 18 Myocardial Cells.

Cells stained either with (a) anti-12CD or (b) α-(sarcomeric) actinin antibody show a similar immunohistochemical Z-line pattern. The fact that the MSD+ NCAM are localized at Z-discs in both single cell and in cells involved in cell-cell contact suggests the possibility of involvement of this NCAM in cell-ECM interactions rather than in cell-cell interactions.

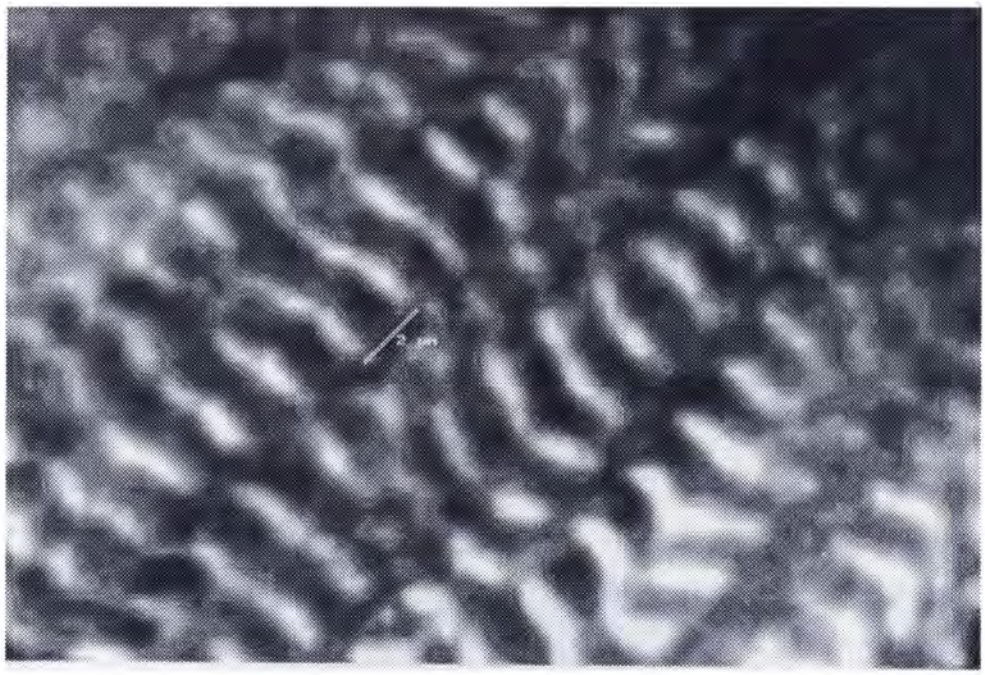

Figure 20. Immunohistochemistry of the Cultured Cardiomyocyte.

Myocardial cells were isolated from the stage 18 embryonic chicken heart. Cells were cultured, fixed, and stained by an anti-12CD. A highly magnified confocal microscopic image shows that the antibody precisely depicts arrays of Z-disc actin stripes at the cell surface. The rib-like stripes with a 2 μm distance detected by the anti-12CD is also typical of cytoplasmic Z disc actin bands. Size bar = 2 μm.

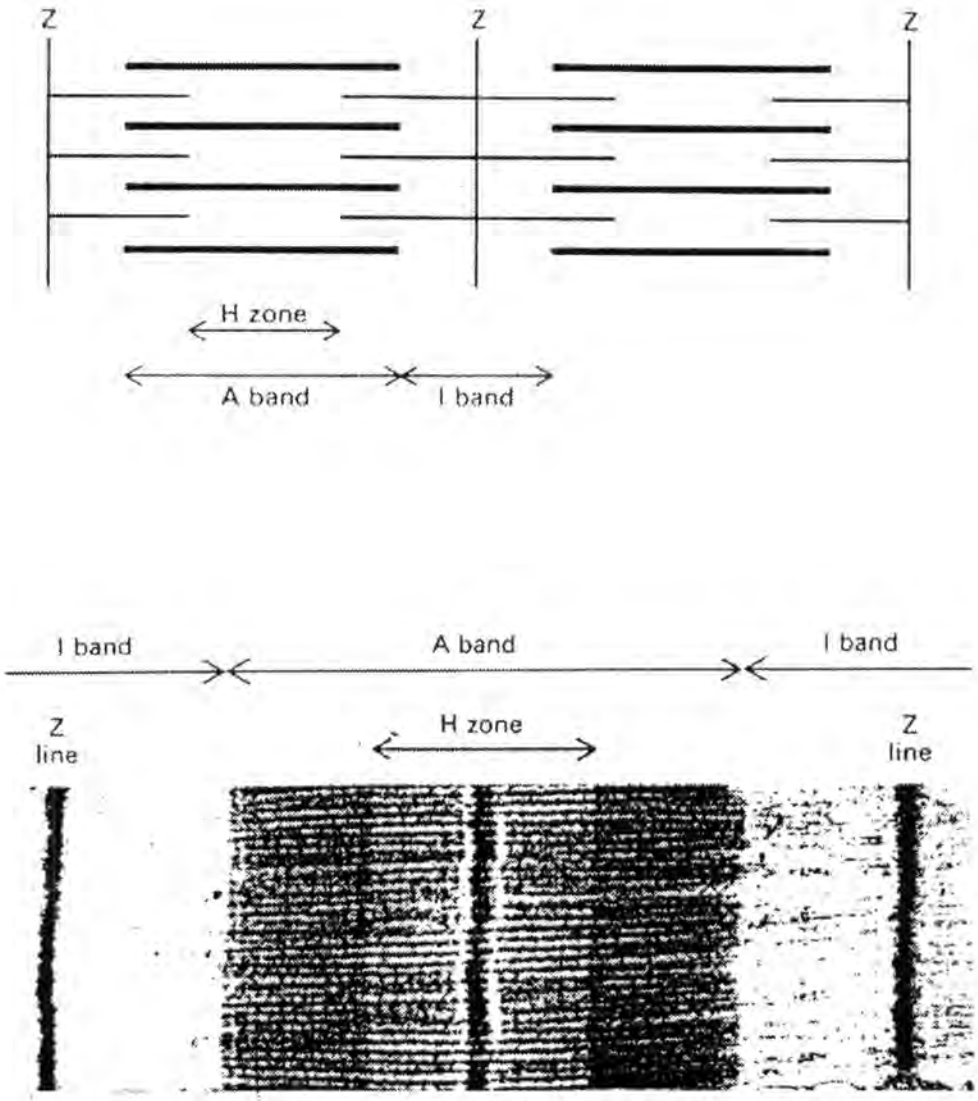

Figure 21. Composition and Alignment of Myofibers in Muscle Tissue.

The upper figure illustrates a basic structure of skeletal muscle, which is shown as an actual electron microscopic image in the lower picture. Muscle is mainly composed of two myofiber types, actin (thin line) and myosin (thick line). Muscle contraction is achieved by the sliding motion of actin filaments into myosin, which results in the shortening of inter-actin distance. The Z-line represents the actin backbone which supports and maintains the movement of myofilaments.

Figure source: L. Stryer, 1988. Biochemistry, 3rd ed.

When this pattern was further observed at a higher magnification, each stripe appears to coincide with the Z-discs that closely underlie the cell membrane. In addition, the inter Z-disc distance that is typically about 2 μm in the mature myofiber (23) precisely matches the distance between MSD-containing NCAM stripes (Figure 20). The typical structure and composition of a myofiber is illustrated in Figure 21. Moreover, the fact that this staining pattern is exhibited by isolated cells (Figure 19) suggests that the maintenance of this pattern may not depend on cell-cell adhesion. The NCAM subspecies containing the MSD region may rather participate in cell-extracellular matrix adhesion rather than cell-cell adhesion.

Because previous studies of costameres were performed later in development (embryonic day 7 or later), we have prepared cardiomyocyte culture which represents the early or late stages of myofibrillogenesis. Myocardial cells are isolated from the stage 18 heart ventricle as described before. When these cells are observed early (day 1 or 2) in culture, the MSD-containing NCAM species are exclusively colocalized at Z-discs (Figure 22, b) together with α-actinin (Figure 22, b). On the other hand, other molecules known to be associated with Z-discs later in development, β1 integrin (Figure 22, c), vinculin (Figure 22, e), or N-cadherin (Figure 22, f), are not yet found at Z discs. Vinculin finally associate with Z discs later in culture (day 4) showing the typical striated stripe pattern (Figure 23, d). While previous immunohistochemical studies have presented rather a complete picture of

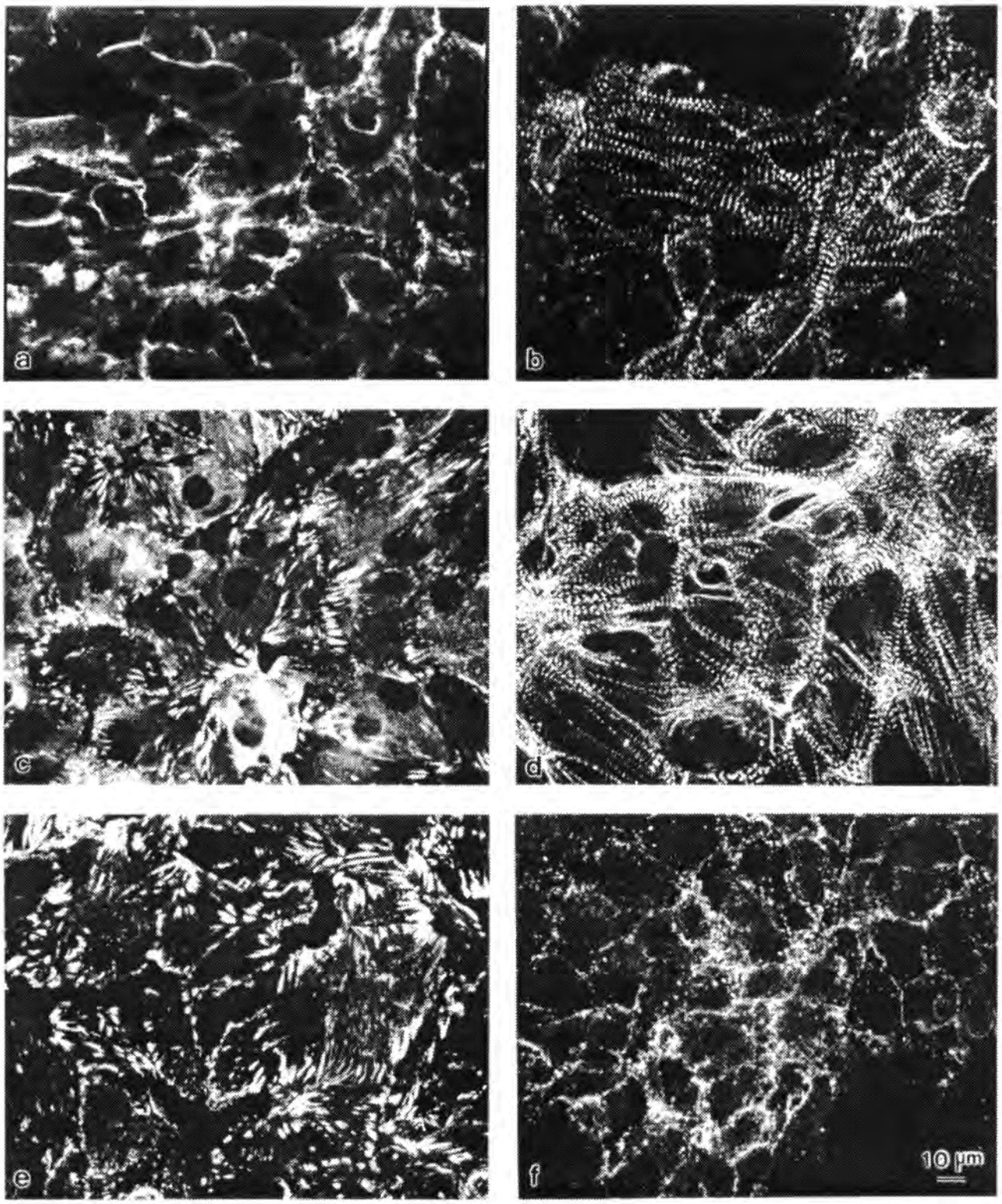

Figure 22. Distributions of Adhesion Molecules and Cytoskeletal Proteins in Cultured Stage 18 Myocardial Cells.

Cardiomyocytes are stained with the following antibodies: (a) anti-pan NCAM, (b) anti-12CD, (c) anti-β1 integrin, (d) anti-α-(sarcomeric) actinin, (e) anti-vinculin, and (f) anti-N-cadherin. Results show that the MSD+ NCAM are the only CAM colocalizing with α-actinin at Z-discs during the early assembly of myofibers in vitro. Size bar = 10 µm.

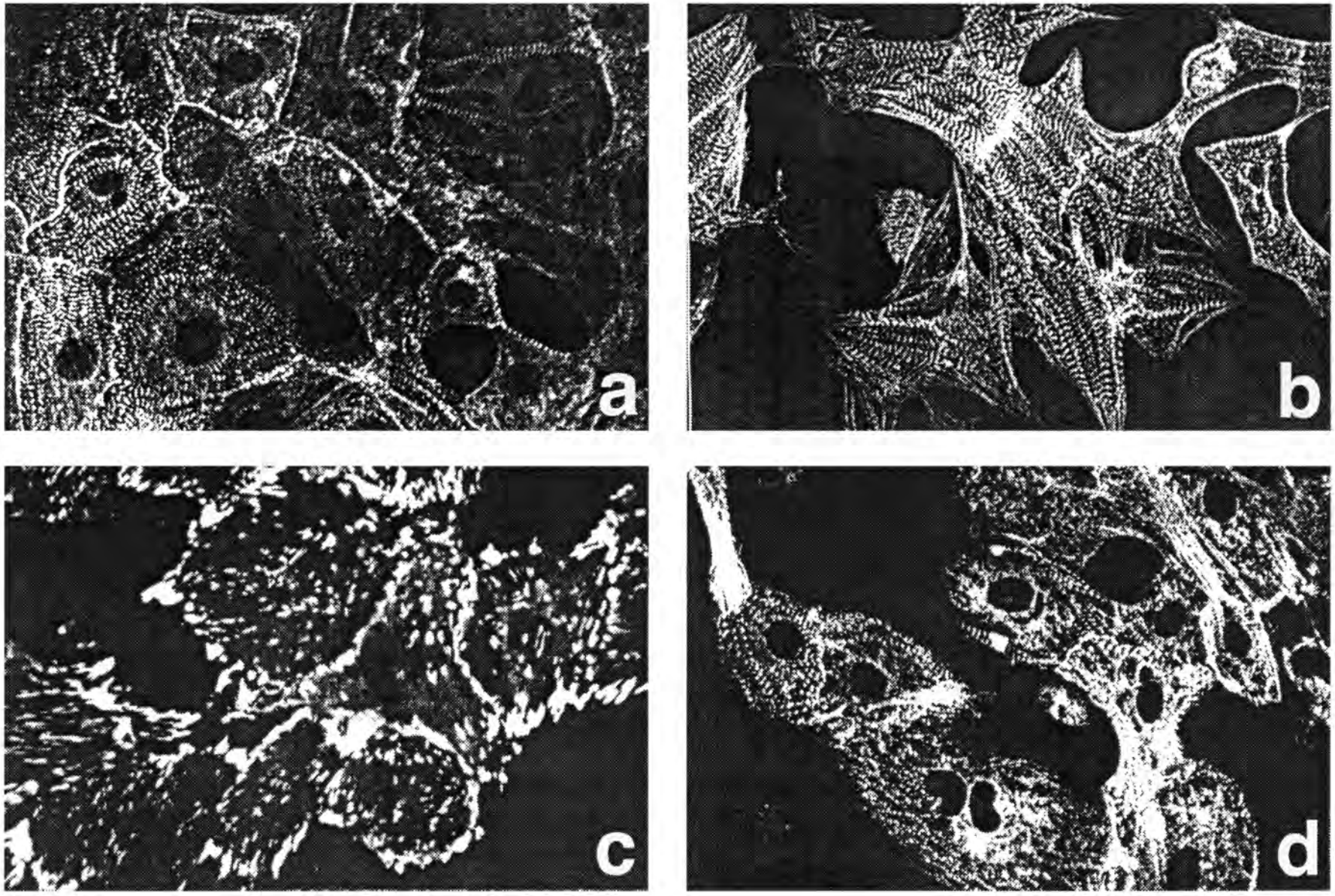
a
b
c
d

Figure 23. Immunohistochemistry of Cultured Myocardial Cells.

Cardiomyocytes are isolated from stage 18 chicken heart, cultured for 1 or 4 days, and immunostained for various structural components of Z-disc. At cuture day 1, cells are detected by (a) anti-12CD, (b) anti-α-(sarcomeric) actinin, or (c) anti-vinculin. Cells are also detected by (d) anti-vinculin at the culture day 4. Note that the MSD-containing NCAMs are colocalized at the Z-disc earlier than vinculin.

costamere structure which contained many cytoplasmic elements including vinculin, actinin, and talin, our data indicates that a supposedly essential element (vinculin) is missing from the costamere at stage 18 whereas the MSD-positive NCAM is already present. Thus, our study indicates that the MSD-containing NCAM has become localized to the Z-disc region even before one of the supposedly essential structural element of costameres (i.e., vinculin) is assembled.

DISCUSSION

Research Scheme to Study NCAM Containing MSD Region

The NCAM gene is composed of a large transcriptional unit including 19 major exons (Figure 2). Three major forms of NCAM are detected in tissues; the ld, sd, and ssd (Figure 1) variants that differ in their association to the plasma membrane. This structural diversity of NCAM polypeptides is generated by an alternative RNA splicing mechanism (56, 72, 79). Overall, three major forms of NCAM have been well identified in brain, heart, and muscle tissues.

Heart, skeletal muscle, and skin NCAM molecules contain a MSD insert in the extracellular portion, while this insert is not present in brain NCAM (15, 43, 51, 72, 79). We have been particularly interested in studying the expression of MSD insert which is spliced into the NCAM protein by a tissue-specific mRNA splicing. The MSD insert is composed of 31 amino acids which are encoded by four additional small exons, A-D, located between exon 12 and 13 of the NCAM gene (Figure 3).

In order to detect the MSD sequences contained in NCAM polypeptides, we have raised two polyclonal antibodies: one is anti-12AB

directed against the polypeptide encoded by exons 12A and B, the second is anti-12CD against the polypeptide encoded by exons 12C and D. We have divided the MSD into two regions because the 3' half of MSD shares higher homology across species than the 5' half; while the sequence of four small exons is highly conserved between humans, rats, and mice, two of the four chicken exons (12A and 12B) are very different in sequence from their mammalian counterparts (51). Therefore, two anti-MSD antibodies, each directed against a specific sequence, would be used for a cross-species study. In addition, these two antibodies would be able to detect if the microscale mRNA splicing occurs between exons 12 A-D. Some studies suggest that only exons 12A and 12D are expressed in the brain from the MSD region (26, 55, 64), whereas that all four of these small exons (12A-D) are expressed in heart, skeletal muscle, and skin (15, 43, 51).

Based on studies using the MSD-specific antibodies, I have made the following novel observations: 1) During heart development, all three splice variants of NCAM (ld, sd, and ssd) that differ in their mode of attachment to the plasma membrane are expressed both in forms containing and in forms lacking the MSD region. 2) At the protein level, the MSD region encoded by exons 12A, 12B, 12C, and 12D appears either to be absent or to be expressed in its entirety, i.e. we see no evidence for alternate splicing among these four small exons. 3) The six forms of NCAM detected in this study each change dramatically in their expression during development. In particular, the percentage of the ld, sd, or ssd form expressed that is also MSD region-positive

can vary nearly between 0 and 100 % in a temporally and spatially controlled manner. 4) At the immunohistochemical level, forms of NCAM containing the MSD region are specifically associated with Z discs early in heart development suggesting a role for this region of NCAM in myofibrillogenesis. 5) In cultured cardiomyocytes, the MSD-containing NCAM appear to be the earliest cell adhesion molecule that is associated with Z discs. This suggests that MSD-expressing NCAM plays a unique role in the cell membrane and myofiber interaction at very early stages of muscle differentiation.

MSD is Present in Many NCAM Splice Variant Forms

Previous studies on forms of NCAM expressed in the developing chicken heart revealed 150, 140, and 130 kD polypeptides (30, 51). The 130 kD polypeptide was found to be GPI-linked to the plasma membrane and to be the predominant form of NCAM in adult heart while the 150 and 140 kD forms were predominant earlier in development. Those studies also have shown that the 180 kD and 140 kD transmembrane forms and the 120 kD GPI-anchored form are present in neural tissue. Particularly, the 180 kD species was believed to be neural tissue-specific and has never been identified in other tissues (30).

In the present study, we have been able to resolve and unambiguously identify six forms of NCAM during heart development (Table 2). 185 and 180

kD polypeptides have been shown to be variants of the ld (large cytoplasmic domain) form of NCAM because they are recognized by monoclonal antibody 4d. 155 and 145 (previously referred to as 150 and 140) kD polypeptides are variants of the sd (small cytoplasmic domain) form of NCAM. 125 (previously referred to as 130) and 120 kD polypeptides are variants of the ssd (small surface domain) form of NCAM as evidenced by the fact that they are released from membranes by PIPLC (Table 1). In each of these pairs, the higher molecular weight forms (185, 155, and 125 kD) are recognized by anti-MSD region antibodies, while the lower molecular weight forms (180, 145, and 120 kD) are not recognized by these antibodies. Thus, each splice variant of NCAM that differs in its attachment to the plasma membrane can also be expressed either containing or lacking the MSD region (Table 2). This interpretation of the identity of NCAM polypeptides is in agreement with the studies of Yoshimi et al. (78) on the 155 kD and 145 kD forms of NCAM expressed during skeletal muscle development.

In previous studies of forms of NCAM expressed during heart development, the ld form observed was considered likely to be of neural origin. The current study suggests, however, that the ld form of NCAM is also expressed by heart tissue. First of all, the 185 and 180 kD forms of NCAM are present at significant levels even in stage 15 (unpublished data by V. Mironov: personal communication) chicken embryos, and these forms peak in expression in 7 day embryos (Figure 14). If these forms of NCAM were expressed by the cells that innervate the heart, they might be expected to

appear later and to be maintained throughout development. Second, the 185 kD form of NCAM is recognized by anti-MSD region antibodies even though these antibodies do not recognize any form of NCAM in the brain. Assuming that the innervation of the heart is similar to the brain in not expressing forms of NCAM recognized by anti-MSD antibodies, then our data would suggest that at least the 185 kD form of NCAM is produced within the musculature of the heart. It is tempting to speculate that the ld form of NCAM may be preferentially expressed in the conduction system, given that the polysialic moieties found on NCAM as well as the carbohydrate epitopes recognized by monoclonal antibody HNK-1 and found on neural NCAM and other neural adhesion molecules are also preferentially expressed in this region of the heart (47, 77).

No Heterogeneity within the MSD Sequence

Data from PCR amplification of cDNAs prepared from developing rat heart mRNAs have suggested that alternative splicing occurs between exons 12A, 12B, 12C, and 12D, and that these four small exons are present together in only a small percentage of NCAM mRNAs (56). Our results provide a very different picture for NCAM molecules at the protein level. Even at the level of CNBr fragments, we see that anti-12AB and anti-12CD recognize the same polypeptide and that there is no apparent heterogeneity in the size of this polypeptide. Because exons 12A and 12D are very small, we may not have

been able to detect their alternative splicing at the protein level; however, we should have been able to detect alternative splicing of exons 12B and 12C because they encode sufficient mass (11 or 14 amino acids) to noticeably affect the migration of a 45 kD CNBr fragment. The PCR data of Reyes et al. (56) indicate that about 70 % of the NCAM mRNA obtained from perinatal rat hearts lacks both exons 12B and 12C and that only 8 to 15 % (depending on whether ld, sd, or ssd molecules are being considered) contains both 12B and 12C. In contrast, at the protein level, we find that for ld, sd, and ssd NCAM, forms of the molecule containing the MSD region represent at least 50 % of the NCAM molecules present in the heart from stage 15 embryos through adults. Moreover, the great majority of NCAM molecules contain the MSD region in 10 and 14-day embryos and in adults. This difference between our results and the results of Reyes et al. (56) may be due to species differences, differences in message stability or the ability of messages to be translated, or to differences in the half-life of the various polypeptides.

Subcellular Localization of the MSD containing NCAM at the Z-Disc

In the heart, we find a surprising difference in the regional expression of forms of NCAM containing and lacking the MSD region in immunohistochemical experiments. Extracts of atrioventricular endocardial cushion tissue included very little NCAM containing the MSD region. Moreover, all the MSD region-containing NCAM in these samples may be

derived from myocardial contamination; only the sd form of NCAM lacking the MSD region is present in Western blots of cultured endocardial and mesenchymal cells (Mironov, V., R.R. Markwald, and S. Hoffman; unpublished observations). In contrast, extracts of ventricle showed equal levels of NCAM molecules containing and lacking the MSD region. Similarly, immunohistochemical analyses showed NCAM molecules containing the MSD region to be present in the myocardium but absent from or present at only low levels in tissues of endocardial origin. Typically, like other molecules involved in cell-cell adhesion, NCAM is known to be expressed uniformly on the cell perimeter at all sites of cell-cell contact throughout heart development. Our discovery on the subcellular distribution of NCAM molecules containing the MSD region is, therefore, even more striking in that the MSD containing NCAM are expressed at sites of cell surface that are distinct from the sites of NCAM species lacking this insert; the MSD-containing NCAM are colocalized with the Z discs of myofibrils which is detected by staining with anti-α-(sarcomeric) actinin. It is clear that the structures recognized by anti-12CD that colocalize with Z discs must be NCAM molecules containing the MSD region because anti-12CD staining is blocked by preincubation of the antibody with the peptide against which it was made or by preincubation of the tissue section with the pan NCAM antibody (data not shown).

We speculate that the structure with which forms of NCAM containing the MSD region are colocalized at the cell membrane is the 'costamere' (48). The costamere is a structure linking the cell membrane and subcellular Z discs and found in the striated and cardiac muscle (12, 34, 63, 73). It is a composite structure containing many cytoplasmic structural elements including vinculin, meta-vinculin, desmin, talin, and α-actinin (34, 63, 73). When these structural components are immunostained, the costamere is visualized as arrays of periodic, rib-like, repeating lattice pattern beneath the cell membrane. A major role of the costamere is to connect the Z-discs of myofibrils to the sarcolemma and to stabilize their association (48). The role of costamere structure appears to be to provide more than a mechanophysical support to the cell. That is, costameres also play a role in transducing extracellular signals into the cells (4).

Recent studies show that cell adhesion molecules mediating ECM-cytoskeleton signalling at the cell surface play important functions in the organization of the muscle contractile apparatus. Adhesion molecules such as αv, α3, β1 integrin and N-cadherin are localized to Z discs while muscle differentiates (4, 23, 29, 37, 44). In vitro inhibition studies demonstrate that elimination of certain cell to cell or cell to matrix interactions on cardiomyocytes using N-cadherin and β1 integrin antibodies result in myofibril disassembly and sarcomeric misalignment in vitro (23, 29). This indicates that i) cytoskeletal contraction is highly dependent on the

organization of actin and myosin, ii) cells respond to changes in the extracellular milieu by dynamically reorganizing the intracellular actin network, iii) cell adhesion molecules transduce signals received from the ECM to the Z disc, and iv) Z discs comprise an essential element of costameres.

Our study, however, does not detect β1 integrin and N-cadherin in association with Z discs at stage 18 when we do detect the MSD-positive NCAM. That is, the MSD-containing NCAM species are already localized at the Z-discs before other structural component of Z-discs (i.e., vinculin) and other adhesion molecules are expressed at the same spot. These observations make NCAM molecules containing the MSD region the earliest-appearing cell-surface adhesion molecule detected in association with Z discs. This leads to our speculation that the NCAM subspecies containing the MSD region may play a key function in the earliest stages of cardiomyofibrillogenesis.

Models for the NCAM Interaction with the Muscle Z-Disc

This study provides two striking insights regarding the role of MSD region-containing NCAM in cellular interactions and in the mechanism of its linkage to the cytoskeleton. 1) NCAM is well known to be a myocardial cell-cell adhesion molecule and is usually observed at sites of cell-cell contact in immunohistological studies. The highlight of our study is that we find that

the MSD-containing NCAM subspecies are uniquely associated with Z-discs. Therefore, our observations suggest that MSD NCAM plays a special role in the mechanical integration of muscle contractile components with the cell membrane. Although NCAM is usually involved in cell-cell adhesion, our observations raise the possibility that cell-cell adhesion does not play a role in the colocalization of MSD region-containing NCAM with Z discs. In vivo, MSD region-containing NCAM is localized in register with Z discs and not in a uniform distribution on the cell surface or in intercalated discs as would be expected for a molecule involved in cell-cell adhesion. In vitro, MSD region-containing NCAM can become colocalized with Z discs in isolated cells where little, if any, of the cell surface is involved in cell-cell contact. These observations raise the possibility that MSD region-containing NCAM is involved in cell-ECM adhesion. This would be consistent with scanning electron microscopic studies that show collagen-containing 'struts' of connective tissue that interact with the myocardial cell surface in register with Z discs (5). Previously, Walsh (75) has recognized the importance of MSD located in a hinge region of NCAM polypeptide. He speculated that the inclusion of a small MSD insert would transform the existing NCAM into a more flexible structure. This indicates that such a change may affect the binding specificity of NCAM (75). 2) NCAM molecules containing the MSD region are identical in the primary structure of their cytoplasmic region with NCAM molecules lacking the MSD region. Nevertheless, the MSD region-containing molecules appear to interact with the cytoskeleton in a manner

that results in their colocalization with Z discs while molecules lacking the MSD region do not interact with the cytoskeleton in this manner. This suggests two models depicted in Figure 24. Either the interaction of MSD region-containing NCAM with an ECM ligand alters the conformation of the cytoplasmic tail of NCAM resulting in its ability to bind to a component of Z discs (Figure 24, A) or MSD region-containing NCAM molecules can interact with a protein in the same cell (cis ligand) that possesses the ability to bind to a component of Z discs (Figure 24, B). Of course, more complicated models are also possible; for example, the interaction of MSD region-containing NCAM with a cis ligand might induce the ability of the NCAM or of the cis ligand to interact with Z discs. Whether MSD region-containing NCAM interacts with an ECM ligand or a cis ligand, the interactive portion of the MSD region may lie in its amino-acid sequence or in the O-linked carbohydrate [recognized by peanut lectin following neuraminidase treatment of the molecule (75)] associated with the MSD region.

Based upon the restricted localization of MSD-containing NCAM at Z-discs observed in heart tissue as well as in isolated cardiomyocytes, we propose that this NCAM species play a role in muscle tissue differentiation. More specifically, the fact that MSD-containing NCAM colocalizes with the Z-disc at very early stages of myofibrillogenesis suggests that this form of NCAM serves as a structural backbone for the molecular assembly of Z-discs. We also speculate that this MSD-containing NCAM plays an active role in

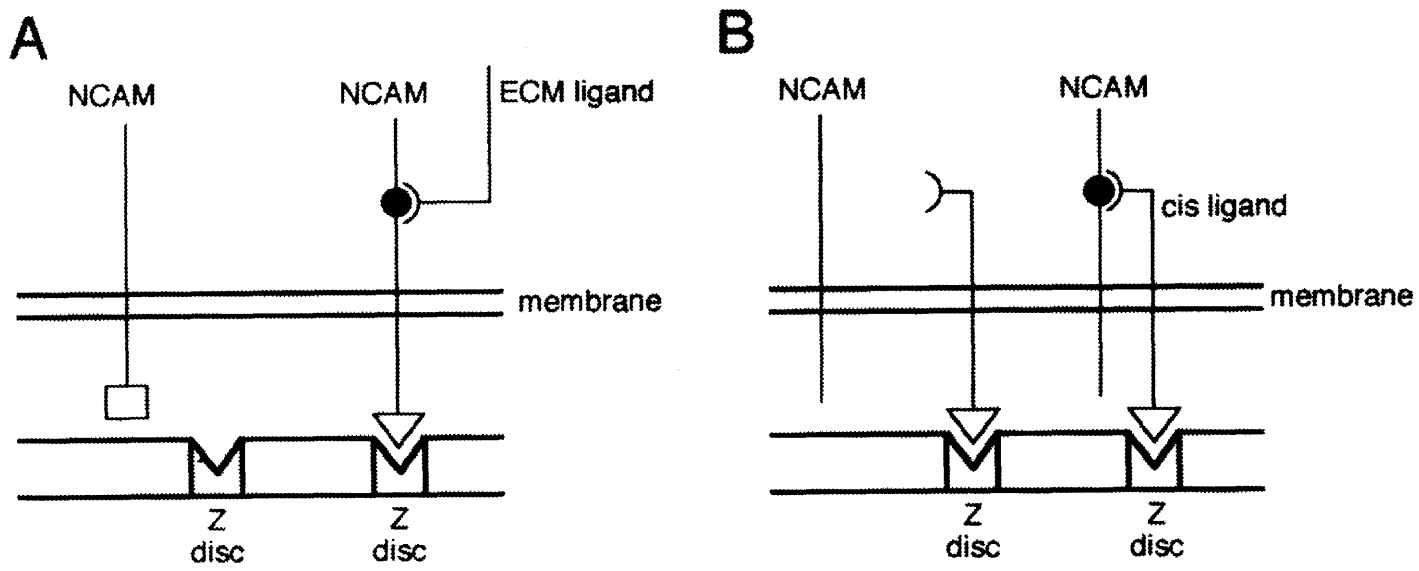

Figure 24. Models for the Mechanism whereby NCAM Containing the MSD Region is Specifically Associated with Z Discs.

The MSD region and its covalently attached O-linked oligosaccharide is represented by a filled circle. Ligands recognizing the MSD region are depicted with a complementary shape. In model A, the interaction of an ECM ligand with the MSD region induces a conformational change in the cytoplasmic tail of NCAM allowing it to bind to the Z discs. NCAM molecules lacking the MSD region cannot bind this ECM ligand and therefore cannot undergo the conformational change that allows them to dock in Z discs. In model B, a cis ligand (present in the plasma membrane of the same cell) binds both to the MSD region and to the Z disc. NCAM molecules lacking the MSD region do not bind to this cis ligand and do not become associated with Z discs.

transmembrane signal transduction via its interaction with components of Z-discs.

The current study demonstrates the feasibility and utility of using antibodies against alternatively spliced regions in NCAM to evaluate their identity and distributions in vivo. The existence of the MSD region in NCAM was deduced from molecular biological studies. However, such studies did not indicate precisely which mRNAs detected on Northern blots using MSD region-specific probes encode which NCAM polypeptides observed on Western blots. Molecular biological studies also did not and cannot indicate the subcellular distribution of forms of NCAM containing the MSD region. The antibodies prepared in the current study have allowed the identification and characterization on Western blots of the forms of NCAM containing the MSD region. Moreover, these antibodies have allowed us to make the striking observation that forms of NCAM containing the MSD region are colocalized with Z discs and thus are likely to be involved in myofibrillogenesis. Antibodies specific for other splice variants of NCAM (e.g. ld and ssd forms and forms containing the VASE insert) may be equally useful for determining the distribution in vivo of particular forms of NCAM and thereby suggesting specialized functions for each of these forms.

Future Studies on the NCAM Containing MSD

In the current study, we have prepared antibodies against two oligopeptides that together comprise the MSD region of chicken NCAM. These antibodies have allowed us to analyze the expression of forms of NCAM in heart development. We find that all ld, sd, and ssd forms of NCAM can contain or lack this MSD insert. During heart development, six various forms of NCAM dramatically change their temporal and spatial patterns of expression. Specially, expression modes of NCAM species containing the MSD sequence are precisely correlated to myofiber differentiation of heart. These findings are reproduced in the cardiomyocyte culture making MSD-positive NCAM the earliest adhesion molecule localized at the Z disc region. We therefore propose new models of NCAM interaction and suggest that the MSD-containing NCAM species play a role in myofibrillogenesis.

Finally, I would like to suggest future follow-up studies for NCAM containing the MSD insert. i) Transgenic or gene knock-out experiment: As for the loss of MSD function, a mouse can be produced via targeted disruption of the MSD sequence in NCAM (knock-out). Likewise, a knock-in mouse with over (redundant)-expressed MSD in NCAM species can be created. These studies would be invaluable in understanding the role of MSD. ii) The mechanism of MSD splicing: Though the MSD expression is highly tissue-specific, how its mRNA splicing is regulated at these tissues is not known. This study would be important in understanding the molecular genetics and the diversity of NCAM .

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